ABSTRACT

Title of Thesis:	THE EFFECTS OF EMBRYONIC NOREPINEPHRINE ON JAPANESE QUAIL BEHAVIOR AND NEUROPHYSIOLOGY
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Stress in poultry breeding flocks results in elevated *in ovo* monoamines affecting behavior and physiology. We injected Japanese quail (*Coturnix japonica*) eggs with 10 μl of 0.05M (n = 111) or 0.01M (n = 113) concentrations of norepinephrine (NE) or saline (n = 112) at ED1 and incubated with intact controls (n = 78) to observe the influences of elevated embryonic NE on behavior and productivity. We tested developmental memory, tonic immobility, open field isolation behaviors, home cage aggression, and novel conspecific responses. We also measured body weights, egg lay and survival-related behaviors before and after rehoming at sexual maturity. Results indicated dose and age differences between treatments. Norepinephrine birds exhibited variations in stress-coping strategies, decreased productivity, increased consumption frequencies, decreased activity levels, and changes in survival-related behaviors following rehoming. Our data suggest that elevated embryonic NE plays a role in behavioral programming with impacts on poultry well-being.

THE EFFECTS OF EMBRYONIC NOREPINEPHRINE ON JAPANESE QUAIL BEHAVIOR AND NEUROPHYSIOLOGY

by

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Dedication

I would like to dedicate this work to my family, who have been incredibly supportive and understanding, throughout my education and who have patiently and flexibly "anchored the fort" for me when I have needed to focus on my graduate work. I would also like to dedicate my thesis to the future graduate students of the Dennis lab. May your experiences be as fulfilling as mine has been.

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List of Abbreviations

ACTH: Adrenocorticotropic hormone

CNS: Central nervous system

CRF: Corticotropic releasing factor

CSF: Cerebrospinal fluid

DA: Dopamine

DOPAC: 3, 4-Dihydroxyphenylacetic acid

DXL: DeKalb XL

ED: Embryonic Day

EP: Epinephrine

HGPS: High group productivity and survivability

HPA- Hypothalamic-pituitary-adrenal

Hr: Hour

HVA: Homovanillic acid

ICV: Intracerebroventricular

IP: Intraperitoneal

Kg: Kilograms

LC: Locus coeruleus

L-DOPA: L-dihydroxyphenylalanine

LGPS: Low group productivity and survivability

MAOA: Monoamine oxidase A

Mg: Milligrams

MHPG: 3-Methoxy-4-hydroxyphenylglycol

MHPG-SO: 3-methoxy-4-hydroxyphenylethyleneglycol sulfate

Ml: Milliliters

NE: Norepinephrine

Ng: Nanograms

PNMT: Phenylethanolamine *N*-methyltransferase

PNS: Peripheral nervous system

Trp: Tryptophan

Tyr: Tyrosine

TH: Tyrosine hydroxylase

VMA: Vanillylmandelic acid

Wk: Wk

5-HIAA: 5-hydroxyindoleacetic

5-HT: Serotonin

5-HT1B: 5-hydroxytryptamine receptor 1B

6-OHDA: 6-hydroxydopamine

μmol: Micromoles

μL: Microliters

μg: Micrograms

Chapter 1: Norepinephrine Form, Function, and Role in Behavior and Animal Well-being

Advancements in Concepts of Animal Well-being

Over recent years, changes in public perceptions and understandings of animal welfare have led agricultural producers to explore new means of improving animal health and well-being while considering industry restraints, costs, and practicality. The animal welfare movement started to gain momentum in the 1960s with the publishing of the U.K.'s Bramble Committee Report focusing on livestock well-being, the U.S.'s Animal Welfare Act focusing on companion and lab animal well-being, and private publications including Rachel Carson's *Silent Spring* and Ruth Harrison's *Animal Machines*. In the 1980s and 1990s, concern for animal welfare increased, driving research in the early 2000s up to the present involving genetic and environmental alterations that could improve the well-being of production animals. Scientific findings have resulted in shifts in the paradigm of animal well-being and societal assumptions of the needs of domestic animals. Despite variable definitions for animal welfare, the Five Freedoms outlined by the U.K. Farm Animal Welfare Commission have consistently been viewed as a pillar for well-being standards. The Five Freedoms include 1) the freedom from hunger and thirst, 2) the freedom from discomfort, 3) the freedom from pain, injury, and disease, 4) the freedom to express normal behavior, and 5) the freedom from fear and distress (FAWC, 1992). However, the concept of animal welfare now addresses not only the absence of suffering and undesirable conditions but also the presence of "positive" physical and mental states with much consideration given to the animal's natural ethology (Dawkins, 2008; Yeates and Main, 2008; Ohl and Van der Staay, 2012). In continuing to advance the well-being of production animals, focuses should include eliminating negative

states of the animals' existence while promoting positive conditions by capitalizing on natural tendencies and behaviors.

Stress and Well-being in the Poultry Industry

The poultry industry generally focuses on either meat or egg production although some niches within the industry rely on dual purpose birds. In the egg industry, laying hens are selected for high and quality egg production, efficient feed conversion, and survivability. In the meat industry, broilers are selected for rapid weight gain, efficient feed conversion, valuable carcass yield, and reproductive performance (Flock et al., 2005). Balancing high rates of production, overall industry goals include minimizing mortality and losses due to disease, conspecific aggression, environmental factors, and other stresses that may be induced by current husbandry models.

While some stress can be healthy in promoting optimal growth, feed utilization, social structure, disease resistance, and reactions to potential danger, intense and sustained fear can be detrimental (Gross, 1983; Jones, 1987; Bryan Jones and Waddington, 1992). Birds that experience chronic stress, especially early in life, often demonstrate delayed maturation, diminished growth, poor feed conversion, low egg production, reduced egg quality, poor plumage, and increased injury (Hill, 1983; Jones and Hughes, 1986; Hemsworth and Barnett, 1989; Mills et al., 1990). A bird may not always show visible behavioral signs of stress, and productivity is not always an accurate indicator of well-being (Duncan and Dawkins, 1983; Hill, 1983). The animals' ethology and genetic tendencies as well as the environment in which they are housed must be considered in mitigating stress.

Methods of curtailing fearful and aggressive behaviors due to or exacerbated by stress should be as minimally invasive as possible. For instance, as an increasing number of producers move away from traditional battery cage setups to floor management to meet consumer pressure for free-range goods, there is concern for higher rates of feather pecking

and cannibalism among the birds. Beak trimming and desnooding have been instituted by many producers as techniques to reduce injury between conspecifics. However, the procedures can be painful and are subject to welfare concerns (Sherwin and Kelland, 1998; Flock et al., 2005). Although they may be effective in decreasing injury and mortality rates among poultry, beak trimming and desnooding highlight the need to search for better ways to control aggressive responses in birds (Sherwin and Kelland, 1998; Farooq et al., 2013; Hartcher et al., 2015). Less invasive practices benefit not only the animals, but also the producers, who can attain higher yield from fewer mortalities and better quality carcasses with less time and money spent on beak trimming and desnooding.

Changing definitions of welfare as well as advancements in technology will lead to better ways to control unwanted behaviors in response to stress. Concentrations of norepinephrine (NE), a catecholamine and stress hormone involved in the "flight or fight" mechanism, can be measured in order to obtain information related to an animal's internal responses towards stress (Dimsdale and Moss, 1980; Kosten et al., 1987; Morilak et al., 2005). Norepinephrine may serve as a gateway to altering fearful and aggressive behaviors due to of its key role in behavioral programming and modulating stress (Francis et al., 1999). With further research of its implications on behavior and physiology, NE can lead to less invasive ways to improve animal well-being in the poultry industry while increasing production quality and quantity through control of stress responses.

Synthesis and Release of Norepinephrine

Fearfulness and aggression can be regulated through the catecholamine NE, which is implicated in the "flight or fight" response as well as arousal and selective responses to stimuli in animals and humans. $_{L}$-tyrosine, the initial precursor in the pathway to NE synthesis, is synthesized in the liver from dietary phenylalanine by phenylalanine hydroxylase (Deutch and Roth, 1999). $_{L}$-tyrosine is then transported from the liver to

catecholamine-secreting neurons (Cunningham, 2002). Tyrosine hydroxylase (TH) is phosphorylated to catalyze the conversion of $_{L}$-tyrosine to $_{L}$-dihydroxyphenylalanine (L-DOPA). $_{L}$-dihydroxyphenylalanine is converted to dopamine (DA) by $_{L}$-DOPA decarboxylase. If DA is not secreted, NE is formed via catalysis by DA β-hydroxylase with ascorbic acid acting as a cofactor. Norepinephrine may then be converted to epinephrine (EP) by phenylethanolamine *N*-methyltransferase (PNMT) through an *S*-methyl transfer from *S*-adenosyl methionine to NE (Whittow, 1999; Ghosh et al., 2001; Stanford, 2001; Mahata et al., 2002; Trifaro, 2002).

Following upstream neuronal signaling, NE undergoes vesicular exocytosis for release into the synaptic gap. In order to initiate a signal, NE must bind to α- or β-adrenergic receptors. Norepinephrine-responsive α-receptors are divided into α_1 and α_2 subtypes while NE-responsive β-receptors can be divided into β_1 and β_2 subtypes (Ahlquist, 1948). Alpha-1 receptors provide an excitatory response, stimulating smooth vascular musculature to cause vasoconstriction with higher affinity for NE over EP. Alpha-2 receptors inhibit NE and renin release in a negative feedback process with equal NE and EP affinity. Beta-1 receptors stimulate the heart and mobilize fatty acids from adipose tissues with equal NE and EP affinity. Beta-2 receptors produce an inhibitory effect on smooth muscle tissue, leading to vasodepression and bronchodilation with lower NE affinity compared to EP affinity (Lands et al., 1967; Lands et al., 1969; Berthelsen and Pettinger, 1977; Minneman et al., 1981; Molinoff, 1984). Once the signal has been initiated, NE is either taken up by vesicles for storage in the cell or, more frequently, is degraded into 3-methoxy-4-hydroxyphenyglycol (MHPG) or vanillylmandelic acid (VMA, 3-methoxy-4-hydroxylmandelic acid) by monoamine oxidase (MAO) or catechol-*O*-methyl transferase (Reinstein et al., 1984; Stanford, 2001).

Norepinephrine is primarily centralized in the brain and the adrenal medulla. The catecholamine's signal originates in the locus coeruleus (LC) and projects to α- and β-

adrenergic receptors in the limbic and cortical regions to influence cognition and arousal (Kim et al., 2008; NCBI, 2015). While NE cannot cross the blood-brain barrier, it can bind to the receptors in the hypothalamus (HYP), acting as a component of a positive and negative feedback loop to induce or suppress the production of corticotropic releasing factor (CRF) which in turn releases or inhibits adrenocorticotropic hormone (ACTH) release and elevated plasma corticosterone levels. Acting as a hormone directly, NE can be secreted from the adrenal medulla into the bloodstream and moves from the sympathetic nervous system to peripheral synapses, facilitating reactions to stress (Haller and Kruk, 2003).

Presence and Detection During Development

Catecholamines are present early in avian development, producing effects on the nervous system and the gut. Tyrosine hydroxylase, DOPA decarboxylase, and dopamine β-hydroxylase (DBH) are immuoreactive enzymes detectable by formaldehyde-induced fluorescence and can be used to predict the downstream production of catecholamines including NE (Zhang and Sieber-Blum, 1992). In the chick, DOPA decarboxylase is found as early as two days into embryonic development before formation of the sympathetic chain, and can be traced throughout the various stages of nervous system development (Enemar et al., 1965). Primary sympathetic chain formation begins on ED3 and ends by ED4.5 while a secondary sympathetic trunk develops by ED6. Norepinephrine is first directly detected in the sympathetic chain at ED3.5 but remains low until ED6. During this period, it can be traced in outgrowing adrenergic cells into the aortic plexus and interrenal buds leading to adrenal medulla growth (Enemar et al., 1965). Following a peak of DOPA decarboxylase activity at ED6, NE proceeds to dramatically increase between ED7 and ED8 resulting in high concentrations of NE at ED8 and ED9. At ED8, the dorsal midline, which acts as a CNS patterning center, is shown to contain more DOPA decarboxylase than any other part of the embryo, suggesting a prominent site of NE production (Enemar et al., 1965; Currle et al.,

2005). At the ED10 stage, spinal ganglia, involved in relaying sensory information to the CNS, are surrounded by convergent nerve cells with a catecholamine presence as detected by fluorescence (Enemar et al., 1965).

In relation to other catecholamines, NE is shown to be significantly higher in concentration when compared to EP and MHPG levels throughout avian pre-hatch development. Norepinephrine levels further increase at an accelerated rate once the birds are hatched, suggesting a vital role in continued development and cognitive response processes in early life (Revilla et al., 2001). In comparison to adult birds, however, the fetal and post-hatch adrenomedulla contain higher levels of NE while the adult adrenomedulla is characterized by higher levels of EP (Shepherd and West, 1951).

Norepinephrine's Relationship with Other Neurotransmitters and Catecholamines

Norepinephrine acts in conjunction with multiple physiological components implicated in fearful and aggressive responses to stress. Tyrosine (Tyr), DA, and EP are synthesized in the same conversion pathway as NE with related effects on mood and arousal and are often measured along with NE to aid in understandings of the broad biological processes and interactions (Nagatsu et al., 1964; Pohorecky et al., 1969). In addition, serotonin (5-HT) is also frequently measured in fear and aggression studies. Due to the number of its associated receptors and subsequently activated effectors and pathways, 5-HT has been accepted as the primary mediator of inter-male aggression with secondary effects on several other signaling molecules (Nelson and Chiavegatto, 2001). Norepinephrine is closely linked with 5-HT regulation of fear and aggression. In a study by Szabo and Blier (2002), the acute and chronic effects of [(S)-2-[[(7-fluoro-4-indanyl)oxy]methyl]morpholine monohydrochloride] (YM992), a 5-HT reuptake inhibitor and 5-HT2A antagonist were observed. Acute YM992 injection effectively decreased NE firing rate in the LC while

YM992 exposure over the course of two days produced in an increased activation of α-adrenergic receptors. Chronic YM992 application resulted in decreased α-adrenergic receptor sensitivity and gradual decrease of NE neuron firing in the LC, ultimately suggesting a degree of regulation of noradrenergic pathways via effects related to 5-HT concentration.

Serotonin receptor antagonism can have direct effects on noradrenergic function. Antagonism of 5-HT1A and 5-HT1B receptors, for example, increased the NE and EP levels in the HYP of a highly aggressive strain of poultry (Dennis and Cheng, 2012). In a weaning piglet study, feed supplementation with tryptophan (Trp), a 5-HT precursor, resulted in significantly increased hypothalamic 5-HT concentrations and decreased salivary cortisol and plasma NE and EP levels accompanied by decreased aggressive behaviors (Liu et al., 2013). Certain drugs used to regulate mood and emotions in humans, such as olanzapine and quetiapine, also affect multiple receptor systems including the 5-HT, α-adrenergic, histamine (His), DA, and muscarinic receptors simultaneously, emphasizing the need to understand the how the pathways interact with one another (Bymaster et al., 1999; Jensen et al., 2008). In addition to 5-HT, NE studies also frequently observe changes in DA and EP, synthesized in the same biochemical pathway, as well as changes in plasma glucocorticoids. Prior research has suggested that under stressful conditions, DA effects on behavior are compensated by a corresponding decrease in NE (Antelman and Caggiula, 1977). Similar receptor activation overlap from different biological compounds in duplicating processes allows for greater homeostatic control and ensures that vital behaviors are carried out to completion or attenuated under appropriate circumstances.

Heritability and Genetic Alteration

Aggression and fear have a heritable component upon which environmental factors may act, presenting an additional method by which behavior can be modulated at a biophysiological level. For example, a study of the basal cerebrospinal fluid (CSF) levels of

NE, the DA metabolite, homovanillic acid (HVA), and the 5-HT metabolite, 5-hydroxyindoleacetic acid (5-HIAA) showed different concentrations between rhesus monkeys descended from unrelated sires while controlling for environment and lifestyle differences (Clarke et al., 1995). Given natural genetic differences that affect neurotransmitter and catecholamine levels, recent advancements in technology have allowed for direct manipulation of genes that can affect such behaviors, aiding in the creation of genetic lines that highlight the relationship and role of catecholamines in behavioral programming. In a study by Cases et al. (1995), alteration of the monoamine oxidase A (MAOA) gene in a murine model led to the deficiency of MAOA, which breaks down 5-HT and NE. Deletion of the MAOA gene increased concentrations of 5-HT, NE, and DA in whole brains. Changes in neurotransmitter and catecholamine levels were accompanied by heightened expression of aggressive and fearful tendencies, showing a direct relationship between genotype and phenotypic expression of neurotransmitter and catecholamines levels with implications for behavior.

In a poultry aggression heritability study by Cheng and Muir (2007) "high group productivity and survivability" (HGPS) birds were genetically selected for based on group-selected high egg productivity and low mortality associated with cannibalism and flightiness and "low group productivity and survivability" (LGPS) birds were genetically selected from the same starting line for low egg productivity and high mortality associated with cannibalism and flightiness. LGPS birds demonstrated significantly higher 5-HT, DA, and EP blood concentrations as well as a higher EP to NE ratio although there was no significant difference in the NE blood concentration. The HGPS line, meanwhile demonstrated significantly higher blood corticosterone levels (Cheng and Muir, 2007). While there were no significant differences in NE concentration between the poultry lines, NE is known to frequently interact with 5-HT, DA, and EP under varying conditions. The data therefore suggest that further

study may be needed to clarify the role of NE in modulating productivity and survivability based on changes in interacting monoamines and glucocorticoids. When compared to a parental aggressive commercial poultry line, DeKalb XL (DXL), the LGSP exhibited a differences in basal EP concentration and plasma EP decrease in response to 5-hydroxytryptamine receptor 1B (5-HT1B) antagonism, indicating some heritable differences even within similar behavioral groups (Dennis, 2009).

Stress-coping mechanisms also have some basis in genetics although significant differences in catecholamine levels are isolated to the central nervous system. When birds from the HGPS line as well as the DXL aggressive line were placed under stress by doubling group size from 4 birds to 8 birds per pen, no significant differences were measured in the peripheral 5-HT, EP, NE, and DA (Cheng and Fahey, 2009). However, differences were seen in brain concentrations between the lines, further supporting a heritable link between neurotransmitters and phenotypic aggression. The raphe nuclei, which contain high densities of serotonergic nerves and afferents that project to the LC, demonstrated an increase in the 5-HT metabolite, 5-HIAA:5-HT ratio for the HGPS control compared to the DXL control. This higher ratio indicates better 5-HT turnover based on elevated levels of the metabolic product versus the metabolic input showing high rates of 5-HT breakdown. Epinephrine was greater in DXL hens while the HGPS exhibited reduced DA turnover as evidenced by a lower levels of 3,4-Dihydroxyphenylacetic acid (DOPAC), a DA metabolite. In both lines, the 5-HIAA decreased while 5-HT concentrations remained the same in the HYP, signifying a lower 5-HT turnover rate and different extents of effects on parts of the brain (Cheng and Fahey, 2009). Further research by Dennis and Cheng (2011) involving birds from the same LGPS, HGPS, and DXL lines showed that dopaminergic manipulation of aggression via DA acting on D1 and D2 pathways is affected by heritability. While D1 agonism increased aggression in all strains, D2 agonism only increased aggression in the HGPS strain demonstrating differences

in activation mechanisms (Dennis and Cheng, 2011). Within strains, there is variation between subordinate and dominant birds as well (Dennis, 2009). In the high aggression strains, subordinate birds demonstrated an increase in plasma NE and an increased NE:EP ratio associated with higher levels of stress while dominant birds exhibited increased hypothalamic NE and EP concentrations but decreased peripheral concentrations. Dominant birds in the low aggression strain, on the other hand had a higher plasma EP concentration (Dennis, 2009). Data thus suggest that physiological expression of fear and aggression may be heritable but can also be further affected by environmental stimuli acting upon the given genotype.

Poultry well-being can be improved by considering environmental factors that can interact with genetics predisposing birds to fearfulness and aggression. These environmental factors may include physical surroundings, social groupings, or diet (Jones and Waddington, 1992; Miller and Mench, 2006). Further study of how the combined effects of genetics and environment on NE pathways and mediation of stress responses could offer insight into behavioral programming to decrease cannibalism and injurious feather-pecking in the industry and promote positive states of well-being.

Norepinephrine Concentrations Related to Fear and Aggression

Norepinephrine concentrations in the body and brain are linked to levels of fearfulness and aggression across species, especially in conjunction with related catecholamines and neurotransmitters (Lamprecht et al., 1972; Tsuda et al., 1988; Tanaka et al., 1998). However, the exact mechanisms driving NE fluctuations centrally in the brain and peripherally in the blood and how those fluctuations translate into fearful and aggressive responses is not fully understood with many conflicting results across studies (see Haden and Scarpa, 2007 for a review). While it is generally accepted that a peripheral increase in NE

accompanies responses to stress, overarching findings throughout the literature suggest that slight increases in NE elicit aggressive responses while slight decreases in NE mitigate aggression (Hegstrand and Eichelman, 1983; Cai et al., 1993). Extreme decreases in NE elicit aggressive responses caused by rapid depletion while excessive amounts of NE compensate for NE depletion, minimizing acts of aggression (Ozawa et al., 1975; Hahn et al., 1982). It has also been suggested that NE follows a biphasic response in which concentrations increase following a stressor then decrease as aggressive acts are performed as part of a noradrenergic reward pathway (Haden and Scarpa, 2007).

Rats were restrained for 10 minutes in a supine position to induce stress with individuals in one group permitted to perform a simulated act of aggression by biting a stick in order to alleviate stress and individuals in the other group not provided with any means of alleviating stress in a study by Tsuda et al. (1988). At 0 minutes and 50 minutes post-stress, the rats were sacrificed and concentrations were determined for plasma corticosterone as well as brain NE and 3-methoxy-4-hydroxyphenylethyleneglycol sulfate (MHPG-SO4), an NE metabolite. At 0 minutes, concentrations did not vary between the groups or a control group that was not placed under stressful conditions. At 50 minutes, data indicated that rats that were allowed to perform the aggressive act of biting as a means of alleviating stress had normalized plasma corticosterone levels though rats that could not bite continued to exhibit elevated concentrations of corticosterone. MHPG-SO4 levels, signifying a breakdown of NE post-stress, had significantly increased in the HYP, amygdala (AMYG), thalamus (THAL), midbrain, basal ganglia, hippocampus (HIPP) and cerebral cortex in biting and non-biting rats but were particularly high within the HYP for the biting rats indicating a higher NE turnover and more rapid return towards the resting state. Another study by Tanaka (1999) demonstrated that rats that were not permitted to perform aggressive acts following a foot shock or immobilization stressor developed more severe gastric lesions and more marked NE

increases in the AMYG compared to rats that were permitted to perform aggressive acts following the stressor. If such acts of aggression are accepted as manifestations of exaggerated stereotypies, data suggest a possible reward pathway implicated in aggressive responses to stress (Tanaka, 1999).

Varying situations invoke stress, fear, and aggression responses that alter norepinephrine concentrations in different ways. Under acute stress, NE increases and causes sensitization followed by rapid responses in several regions of the brain including the HYP, THAL, AMYG, HIPP, striatum, pons-medulla oblongata, and prefrontal cortex (Tanaka et al., 1982; Southwick et al., 1999). Chronic stress alters physiological mechanisms related to catecholamine production although findings have been somewhat conflicting in how concentrations are altered. Dunčko, et al., (2001) showed that under chronic stress, mRNA expression of TH in the LC decreases in a rat model, suggesting diminished activity in the conversion pathway between Tyr and NE. However, as a protective adaptation that may serve to minimize catecholamine depletion, other studies have shown that TH synthesis in the HIPP and LC of rats increases while basal NE concentrations remain the same (Nisenbaum et al., 1991; Melia et al., 1992). Locus coeruleus neuron sensitivity to excitatory stimuli increases under prolonged stress, often resulting in excessive and exaggerated catecholamine responses (Simson and Weiss, 1994; Southwick et al., 1999). Fearful behaviors may thus be the result of depleted NE concentrations in certain regions of the brain despite higher concentrations in the blood and the brain overall.

Types of stresses may affect the extent to which NE is released. Physical stresses, such as immobilization and tail shocks to rats appear to produce greater NE increases in extended brain regions whereas as psychological and emotional stresses and conditioned fear result in more NE release in the LC, HYP, and AMYG. Predictability of physical stresses mitigates NE release while predictability of psychological stresses increase NE release

highlighting the implications that repeated or sustained psychological stress can have on long-term physiology (Tanaka, 1999). In addition, other factors such as how the stress is delivered in time and the demographics of the subject, including age alter NE concentrations. Periods of stress interrupted by rest periods result in greater NE release than uninterrupted stress periods. Older rats subjected to shocks or immobilization took a longer time to recover from NE release than younger rats (Tanaka, 1999). Norepeinephrine release and breakdown is dependent on type of stress, duration of stress, and subject demographics.

Maternal Stress

It is well-documented that incubation stress and maternal stress are linked to hypothalamic-pituitary-adrenal (HPA)-axis dysregulation and have a significant impact on catecholamine levels and long-term behavior of offspring although the precise mechanism by which behavioral responses to stress are altered is unknown (Peters, 1986; Van den Bergh et al., 2008). Multiple mammalian and avian studies have associated maternal stress with an impaired coping ability and an increased incidence of affective disorders within subsequent generations. In humans, adult children of women who have reported physically and/or emotionally stressful pregnancies exhibit higher rates of PTSD, anxiety, ADHD, and depression (Watson et al., 1999; Brown et al., 2000; Talge et al., 2007; Kinsella and Monk, 2009). In murine models, offspring of stressed rats have demonstrated increased depressive tendencies and learned helplessness in novel environment and fear-induction behavioral tests compared to offspring of unstressed mothers (Thompson, 1957; Fride et al., 1986; Fride and Weinstock, 1988; Alonso et al., 1991; Vallee et al., 1997; Secoli and Teixeira, 1998; Weinstock, 2002; Morley-Fletcher et al., 2003; Green et al., 2011). Rat studies also show that maternal stress results in a feminization of lordosis behavior and no change or a decrease in male copulatory behavior, which may be due to a decrease in plasma testosterone (Dahlöf et al., 1977; Ward and Weisz, 1980; Rhees and Fleming, 1981; Ward, 1984; Love et al., 2008).

Physically, offspring of rats stressed by overcrowding and saline injections during pregnancy demonstrate a decrease in 5-HT and synaptic density in the HIPP but an overall increase in fetal brain Trp, 5-HT, and 5-HIAA linked to an increase in maternal plasma free Trp (Peters, 1986; Hayashi et al., 1998). Prenatal stress can lead to reduced body weight, preterm births, and increased mortality (Rhees and Fleming, 1981; Newton and Hunt, 1984; Wadhwa et al., 1993; Dole et al., 2003; Rondo et al., 2003; Grote et al., 2010; Nkansah-Amankra et al., 2010).

In avian embryonic models, maternal and incubation stress can result in catecholamine and hormone fluctuations and developmental impacts (Epple et al., 1992; Hayward and Wingfield, 2004). Norepinephrine, EP, and DA are naturally occurring during embryonic development (Enemar et al., 1965). However, stresses on the egg including initial input, asphyxiation, handling, and cooling at certain stages during incubation can increase catecholamine plasma and allantoic levels (Epple et al., 1992; Epple et al., 1997). Incubation stressors may impact aggressive behavior as well. Increased incubation heat in developing chicken eggs or injection of 60 ng of corticosterone at ED16 can result in decreased pecking aggression in adult treatment males compared to control males while there is no significant difference in behaviors of adult hens (Lay and Wilson, 2002). Maternal stress can also alter *in ovo* hormone levels. Maternal corticosterone levels elevated during egg lay in wild and domestic birds due to poor environmental nesting conditions, predator-based fear, and articial implants placed under the skin result in higher yolk concentrations of corticosterone (Hayward and Wingfield, 2004; Saino et al., 2005; Love et al., 2008; Schoech et al., 2011). Offspring of avian mothers with elevated corticosterone levels have a lower hatchability rate, body weight, and plumage condition and exhibit increased HPA axis activity in response to restraint and handling stresses (Hayward and Wingfield, 2004; Saino et al., 2005; Love et al., 2008). Overall evidence suggests that developmental stresses may result in increased *in ovo* catecholamine and hormone levels, including NE, that impact HPA-axis formation and long-

term affective states and behaviors in avian species similar to some, but not all, effects seen in mammalian models of maternal stress (Lay and Wilson, 2002).

Effects of Exogenous Norepinephrine and Tyrosine Delivery

Although numerous drug studies have been conducted using NE agonists and antagonists to observe the effects of changing endogenous NE concentrations, there has been little focus on the effects of direct exogenous administration of NE or Tyr. Research that has been conducted has frequently centered on the role of exogenous NE and Tyr in mediating fear and aggression. In a study by Thompson et al. (1974) intraperitoneal (IP) injected doses of NE in the range of 0.05 mg/kg to 0.2 mg/kg did not show a significant difference in levels of fearfulness in adult chickens as measured by tonic immobility (TI) duration (Thompson et al., 1974; Forkman et al., 2007). However, a repeated study in adult chickens using NE in higher doses of 0.125 to 1.0 mg/kg did produce a significant difference in the TI duration of chickens. Subjects demonstrated longer durations of TI indicative of greater sympathetic stimulation and more fear at higher doses of NE (Thompson and Joseph, 1978). These data suggest a threshold at which exogenous delivery of NE produces a behavioral response in an adult animal.

Tyrosine (Tyr) treatment through injections and through dietary supplementation in rat studies, meanwhile, has made stressed subjects less prone to hypothalamic NE depletion and fearful tendencies. Shock-stressed rats that received Tyr via IP injections exhibited significantly decreased fearful behaviors in open field and hole-poke tests. Norepinephrine levels in the LC and HYP were significantly lower in stressed rats that did not receive Tyr compared to unstressed rats that did not receive Tyr, unstressed rats that did receive Tyr, and stressed rats that did receive Tyr. Norepinephrine levels in the HIPP were significantly greater for stressed rats that received Tyr compared to all other treatment groups. Unstressed

rats receiving Tyr injections did not exhibit any significant change in NE levels in the LC, HYP, or HIPP indicating that the respective brain chemistry was only altered by exogenous Tyr under stressful conditions (Reinstein et al., 1984). In a subsequent feed supplementation study, stressed rats that did not receive Tyr demonstrated significantly depleted hypothalamic NE concentrations and significantly increased plasma corticosterone concentrations as well as significantly reduced locomotion, hole-poking, and standing behaviors compared to stressed rats that received Tyr in their feed (Reinstein et al., 1985). These collaborative studies suggest that fearful behaviors are associated with depletion of NE in the brain and increased plasma corticosterone, which may be countered by administration of exogenous Tyr for upstream conversion and ultimate replacement of NE.

Exogenous NE administration has also been shown to have effects on cognition. As part of an adaptive response to stress, release of NE can improve recall of fear-inducing stimuli in order to help an organism mentally encode and react to stressful or dangerous situations in the future (Arnsten, 1998; Markham et al., 2006). However, excessive NE release can translate to impaired memory and lead to depressive responses to stress. Numerous mammalian studies have shown that basal release of NE is necessary for proper prefrontal cortex function and memory processing, but excessive amounts of released NE affect different types of memory to varying degrees (Arnsten, 1998). Injection of NE agonists and antagonists in chicks showed that NE acts upon β_1-adrenergic receptors in the basal ganglia, aiding in short-term memory processing at differing time points in the memory consolidation process, overall enhancing memory (Gibbs and Summers, 2005). In an exogenous administration rat study by Liang et al (1986), injection of a low dose of NE directly to the AMYG after inhibitory avoidance task training resulted in significantly increased retention while higher doses had no effect.

Supplementation with precursors in the NE-synthetic pathway counteracts the negative cognitive effects of dramatic NE depletion under stressful conditions. In a human

model, cognitive function and memory in cadets undergoing military training indicated that Tyr supplementation decreased stress and fatigue in relation to cognitive performance in psychologically and physically demanding environments(Deijen et al., 1999). Conversely, military personnel at the Survival, Evasion, Resistance, and Escape (SERE) School reported increased scores of anger when Tyr was administered in food bars given to the trainees 60 minutes prior to a mock interrogation simulation. However, the Tyr food bars had no effect on reported scores of tension, depression, fatigue, vigor, and confusion and no effect on physiological measurements of heart rate or salivary cortisol levels compared to subjects who received a placebo and underwent the same mock interrogation simulation (Lieberman et al., 2015).

While limited research has focused on the effects of exogenous NE and Tyr delivery in adult animals and humans, even less research has focused on the effects of exogenous NE during development. Quail have frequently been used as a research model representative of all poultry due to their similar attributes but relatively lower cost, space, and time constraints (Wilson et al., 1961). As dual purpose birds, quail are a part of the poultry industry and exhibit similar ontological development to other domestic fowl (Ainsworth et al., 2010). However, some species of quail reach sexual maturity more quickly than larger fowl such as turkeys and chickens, making them an ideal model in long-term developmental studies. In an in vitro study, quail neural crest cells exposed to 10 μM NE in HBSS daily over the course of 15 days demonstrated an increase in adrenergic expression and an increase in catecholamine content of the cells as detected by fluorescence as well as TH and DBH biosynthetic enzymes (Zhang and Sieber-Blum, 1992). Findings suggested that exogenous delivery during key developmental stages leads to higher basal levels of NE post-hatch. Norepinephrine has neurogenic effects in adults and may similarly impact adrenergic receptor development during embryogenesis, altering receptor density and binding affinity to increase or decrease sensitivity as well (Jhaveri et al., 2010; Camp, 2015). A later in vivo bobwhite quail study

injected 0.03M NE into quail eggs at E21 or E22 of a 23 day incubation period. The NE injection instantaneously elevated embryonic heart rates and temporarily elevated heart rates within the first couple of hours post-hatch, showing heightened arousal in the chicks. Norepinephrine injections also resulted in detrimental cognitive effects on perceptual learning and memory with the quail chicks demonstrating a decreased ability to recognize a familiar maternal call compared to control chicks (Markham et al., 2006). Decreased cognitive ability may be due to NE binding to inhibitory α_1-adrenoreceptors once all β-adrenoreceptors are bound (Gibbs and Summers, 2001; 2002; Gibbs and Summers, 2005). Due to the varied immediate and long-term effects that NE has on growth and development based on prior research, findings highlight the need to further investigate the potential effects of altering NE concentrations in embryological states.

Norepinephrine Implications for the Enteric System

Enteric bacteria produce hormones and neurotransmitters, including NE, that crosstalk with host systems via bacterial receptors in the gut, altering microbiome composition and often increasing concentrations of pathogenic bacteria (Galland, 2014). Norepinephrine and other catecholamines act as siderophores to complex with transferrin or lactoferrin, resulting in iron loss. Iron can then be taken up by pathogenic bacteria to support growth (Freestone et al., 2000; Freestone et al., 2002). When NE, EP, DA, and DOPA are added to cultures of gram negative bacteria, *Escherichia coli* (*E. coli*), *Yersinia enterocolitica* (*Y. enterolitica*), and *Pseudomonas aeruginosa* (*P. aeruginosa*) grow significantly faster, with NE producing the greatest increase in growth rate compared to the other neurotransmitters (Lyte and Ernst, 1992).

Culture data translate to *in vivo* findings as well. In a combined *in vitro* and *in vivo* study, several *Campylobacter* strains, which are known to cause illness in humans, were cultured with NE (Aroori et al., 2014). Bacterial strains all demonstrated significantly

increased growth and motility *in vitro* compared to controls cultured without NE, with some of the strains showing increased attack of human T84 epithelial cells and destruction of gap junctions *in vitro*. While orally-gavaged NE-cultured *Campylobacter jejuni* did not demonstrate a higher colonization rate in the ceca chickens, the bacteria did exhibit an enhanced ability to leave the intestines, and a higher rate of the chickens' liver tissues tested positive for the presence of the species (Aroori et al., 2014). In a mammalian *ex vivo* study, cecum were removed from mice then bathed in luminal and contraluminal baths of NE or EP for 15 minutes before a 90 minute exposure to *E. coli* O157:H7. The pathogenic bacteria demonstrated an increased adherence to the cecal mucosa for both neuromodulators (Chen et al., 2003). Norepinephrine pre-treatment also increases *Salmonella enterica* (*S. enterica*) growth, colonization, and systemic spread in mouse and chicken models (Methner et al., 2008). Norepinephrine levels in the gut increase in response to stress and trauma, raising the probability for infection and pathogenicity when an animal is placed in subpar conditions. In a supportive *in vivo* murine study by Lyte and Bailey (1997), IP injection of 6-hydroxydopamine (6-OHDA) to destroy noradrenergic receptors and subsequent blockage with desparimine hydrochloride showed that NE released during simulated trauma can temporarily increase pathogenic gram-negative bacteria, particularly *E. coli*.

Microbiome compositions can modify affective behavioral states both positively and negatively through hormone-mediated HPA-axis communication, especially during critical periods in development (Christian et al., 2015). Probiotics can also alter gut bacterial prevalence with behavioral programming effects related to anxiety and depression (Manco, 2012; Foster and Neufeld, 2013). As probiotic use to improve gut health becomes increasingly more prevalent, the impacts of enteric bacterial communications via NE and other hormones may carry implications for the application of probiotics as a vehicle to program behavior.

Norepinephrine in Relation to Other Health Parameters

At an industry scale, the effects of NE on water and feed intake, especially if delivered through dietary supplementation, are vital to understand in relation to stress as well as productivity. In a study by Denbow et al.(1981), water and feed intake and rectal temperature were measured as indirect parameters of stress and fearfulness after independent lateral ventral injections of 0.21, 0.44, or 0.65 μmol of DA, 0.19, 0.40, or 0.59 μmol of NE, or 0.18, 0.39, or 0.55 μmol of EP in artificial cerebrospinal fluid in 4 wk old chickens. Following NE injection, body temperature significantly decreased among the birds (Denbow et al., 1981). While there were no obvious behavioral indicators of stress in the chickens, in humans, temperature decreases have been associated with stress responses, which may suggest that physiological stress mechanisms were activated by the NE injection (Vinkers et al., 2013). Although no catecholamine significantly affected water intake and only EP significantly increased feed intake, NE did cause some activity depression, which may have hidden any significant changes in feed intake. In rats, earlier studies have shown that NE injection into the HYP and paraventricular nucleus stimulate food consumption in satiated rats (Grandison and Guidotti, 1977; Weiss et al., 1986). Alternatively, administration of L-NE to the perifornical medial HYP and the perifornical lateral HYP of rats suppressed food consumption although D-NE did not (Margules, 1970; Hoebel et al., 1989). Norepinephrine's effect on satiety therefore appears to be location-specific.

Norepinephrine also increases the release of growth hormone (GH). When microinjected into the ventromedial nucleus of the HYP in bonobos, NE significantly increased GH levels blood serum without coinciding increases in blood pressure, heart rate, blood glucose levels, or glycerol(Toivola and Gale, 1972). Agents that deplete NE block the release of GH, indicating an NE-specific effect on GH (Muller et al., 1967; MacLeod et al., 1969). In poultry, differences in blood GH concentrations have been associated with varying growth rates in broilers and laying hens (Harvey et al., 1979) Norepinephrine may thus have

implications for poultry growth, especially if administered during developmental stages pre- and post-hatch within an industry setting. Further research on NE's relationship to developmental in terms of growth would provide insight into its potential effects if used in feeds.

Conclusion

Norepinephrine is inextricably linked to animal well-being concerns through mediation of stress responses. However, research has been somewhat conflicting in identifying the precise mechanism and role NE plays in facilitating and attenuating stress behaviors. As a key modulator of "fight or flight" responses, NE may serve as a gateway to understanding the junction between physiology and behavioral expression when altered during early development. Prior research has demonstrated that maternal stress leading to elevated embryonic hormones and neurotransmitters has short- and long-term impacts on behavior and physiology. Offspring are more likely to exhibit affective disorders, HPA-axis dysregulation, and modified adrenergic receptor networks (Hayward et al., 2005; Talge et al., 2007). Elevated NE resulting from various types of stress in children, adolescents, and adults can also impact future stress-coping abilities and adrenergic density and binding affinity (Liu et al., 2000; Romeo et al., 2006; Bingham et al., 2011). While the general effects of maternal and later life stress have been well-explored, particularly in mammals, little research has been conducted on the effects of specifically NE during early embryogenesis in an avian model. Our research seeks to investigate the influence of NE on poultry behavior and morphology throughout ontogenesis to provide insight into how NE levels may be manipulated in development through internal or external means to program behavior. Results from our study clarify embryonic NE's role in expression of cognitive, fearful, aggressive, social, and survival-related behaviors as well as in productivity and stress-coping mechanisms with implications for poultry well-being and improved commercial management.

Chapter 2: The effects of embryonic norepinephrine on cognitive, fearful, aggressive, and social responses in developing Japanese quail

Abstract

Our research assessed the effects of elevated embryonic NE on affective behaviors through stress-induction tests, concluding that NE impacts cognitive, fearful, aggressive, and social responses in a dose- and age-dependent manner. Maternal stress in poultry breeding flocks can lead to elevated *in ovo* glucocorticoids and monoamines, such as norepinephrine (NE), with long-term impacts on offspring affective states, survival behaviors, and stress-coping abilities in both mammals and birds. To assess the effects of elevated embryonic NE in poultry, Japanese quail (*Coturnix japonica*) embryos were injected with 10 μL of 0.01M (low dose) or 0.05M (high dose) treatment concentrations of NE or saline at ED1 (n = 130) and incubated along with intact controls (n = 80). Memory was measured in a maze at 4 (juvenile) and 8 (sexually mature) wks of age, tonic immobility at 2, 5, and 9 wks, open field isolation responses at 1, 5, and 8 wks, home cage focal observations at 6 wks following rehoming and at 11 wks, and novel conspecific responses at 10 wks. Results showed that juvenile high dose birds had greater first arm latencies than saline birds ($P = 0.03$) and mature low dose birds tended to have lower correct arm latencies than intact birds ($P = 0.06$) but there were no differences in correctness on the first attempt ($P = 0.23$). Low dose chicks tended to exhibit higher TI duration ($P = 0.0733$) but there were no induction differences ($P = 0.8291$). Open field isolation tests indicated a tendency for high dose birds to demonstrate more anxious behaviors including defecation ($P = 0.6166$, $F_{3, 91} = 0.06$) and stationary bouts ($P = 0.0896$). Saline birds foraged more than other treatments ($P = 0.0207$). In home cage focal observations, intact birds demonstrated more gentle feather pecking behavior than other treatments at wk 11 ($P = 0.0576$). High dose birds pecked at a conspecific more than saline

birds treatment (P = 0.0386) and low dose birds tended to attempt to escape more than other treatments (P = 0.0665) in the novel conspecific test. Overall, results provide evidence that elevated embryonic NE alters cognitive, fearful, aggressive, and social behaviors in a dose- and age-dependent manner and reduces stress-coping abilities. These data suggest a role of NE in behavioral programming and indicate the importance of minimizing maternal stress in the poultry industry to mitigate the effects of increased *in ovo* NE.

Introduction

Norepinephrine (NE) is a key catecholamine modulator of fight or flight responses, aggressive and fearful behaviors, and long-term mood alterations in mammalian and avian species. In the poultry industry, fear due to or exacerbated by stress can lead to cannibalism, feather pecking, and piling (Hughes and Duncan, 1972). These behaviors can result in decreased carcass quality and quantity, reduced productivity, and jeopardized bird health and well-being (Hill, 1983; Jones and Hughes, 1986; Hemsworth and Barnett, 1989; Mills et al., 1990; Dennis, 2016). Common sources of stress in the industry include transport, high stockperson turnover, social aggression, changing environmental parameters, and feed restrictions. Due to birds' frequent exposure to stressors and NE's role in regulating stress-related responses, NE may serve as a gateway in behavioral programing, bettering understanding of stress-coping mechanisms in application to the poultry industry in order to improve well-being and productivity (Francis et al., 1999).

As a neurotransmitter, NE is produced in the LC and binds to α- and β-adrenergic receptors in the limbic and cortical regions of the brain (Ahlquist, 1948; Gibbs and Summers, 2002; Kim et al., 2008; NCBI, 2015). Norepinephrine also acts as a hormone secreted from the adrenal medulla into the bloodstream and from the sympathetic nervous system moving into peripheral synapses (Haller and Kruk, 2003). The catecholamine is closely associated with several other hormones and monoamines. Glucocorticoids and NE can act individually

or synergistically through crosstalk to produce responses to stress (Joëls et al., 2011; Krugers et al., 2012). Dopamine (DA) and epinephrine (EP) are produced in the same pathway as NE, and concentration changes of NE during periods of stress are often accompanied by changes in DA and EP due to interactions of the catecholamines (Whittow, 1999; Ghosh et al., 2001; Stanford, 2001; Mahata et al., 2002; Trifaro, 2002). Serotonin (5-HT) interacts with NE as well. Excess NE potentially activates α-receptors on serotonergic nerve terminals to inhibit 5-HT release in the HIPP in a negative feedback loop (Frankhuyzen and Mulder, 1980). Serotonin can be broken down by noradrenergic receptors to a lesser degree and changes in concentration frequently coincide with changes in concentration of glucocorticoids, DA, EP, and NE during periods of stress and aggression (Nelson and Chiavegatto, 2001; Szabo and Blier, 2002; Dennis and Cheng, 2012; Liu et al., 2013). Fluctuations in other glucocorticoids and monoamines can be used to predict changes in NE concentrations in response to stress and aggression.

While research is somewhat conflicting concerning the fluctuation of NE levels under stressful conditions, a review of the literature based on the observed direct effects of peripheral NE and changes in interacting monoamines and glucocorticoids concludes that extreme increases of NE mitigate aggression, slight increases of NE elicit aggression, extreme decreases of NE elicit aggression, and slight decreases of NE mitigate aggression (Table 1; see Haden & Scarpa, 2007 for a review). These patterns of NE fluctuation may be due to how NE is metabolized and how feedback loops participate in communication during NE metabolism. Norepinephrine may follow a biphasic response in which concentrations increase under stressful conditions then decrease following associated aggressive behaviors that activate adrenergic reward pathways (Tsuda et al., 1988; Haden and Scarpa, 2007). Acute and chronic physical and emotional stress, however, has short- and long-term effects on adrenergic pathways and receptor expression. Acute stress increases cortical and subcortical connectivity and responsiveness while chronic stress can lead to adrenergic network

degeneration (Morilak et al., 2005; Kitayama et al., 2008; Buffalari and Grace, 2009; Hermans et al., 2011). According to the catecholamine hypothesis of affective disorders, long-term stress associated with a lack of catecholamines, particularly NE, at receptor sites can cause or exacerbate mood disorders (Schildkraut, 1965).

Table 1. Effects of NE fluctuations on aggression.

Change in Norepinephrine Concentration	**Effect on Aggressive Response**	**References**
Extreme NE Increase	*Aggression Decreases*	*(Reinstein et al., 1984; Deijen et al., 1999)*
Slight NE Increase	*Aggression Increases*	*(Cai et al., 1993)*
Slight NE Decrease	*Aggression Decreases*	*(Lavine, 1997)*
Extreme NE Decrease	*Aggression Increases*	*(Thoa et al., 1972)*

Prevalence of stressors can cause long-term alteration of receptors resulting in constantly poor affective states and increased fearful and aggressive behaviors (Dimsdale et al., 1994; Camp, 2015). Many ways of mitigating stress and improving stress-coping mechanisms have been researched to reduce costs of stress for the poultry industry and bird well-being. Various types of practical environmental enrichment have been successful in redirecting fearfulness and aggression towards natural, healthy behaviors. Types of environmental enrichment include foraging and feeding opportunities (Miller and Mench, 2006), structural components (Jones and Waddington, 1992; Gvaryahu et al., 1994; Newberry, 1995; Sherwin et al., 1999; Miller and Mench, 2006), sensory stimulation (Jones and Waddington, 1992; Newberry, 1995; Jones, 2002; Miller and Mench, 2006), toys (Jones and Waddington, 1992; Gvaryahu et al., 1994; Newberry, 1995), and social interactions (Jones and Waddington, 1992; Reed et al., 1993; Miller and Mench, 2006). Environmental enrichment promotes positive social and beak-related behaviors including foraging, feeding, preening, and gentle feather pecking and decreases detrimental beak-related behaviors including aggressive and severe feather pecking and cannibalism (Bubier, 1996; Hughes and Grigor, 1996).

In addition to environmental enrichment, genetic selection against aggression is an effective means of controlling unwanted behaviors in poultry (Cheng and Muir, 2007; Cheng and Fahey, 2009; Dennis, 2009). “High group productivity and survivability” (HGPS) chickens are group selected based on high egg productivity and low death rates associated with cannibalism and flightiness. “Low group productivity and survivability” (LGPS) chickens, are conversely selected for bases on low egg productivity and increased aggression. LGPS birds demonstrate significantly higher 5-HT, DA, and EP blood concentrations and a higher EP to NE ratio but no significant difference in NE blood concentrations (Cheng and Muir, 2007). However, within high and low aggression lines of poultry, environmental factors such as dominant and submissive tendencies can play a role in physiological and behavioral responses to stress with differences in catecolamine receptor activation between dominant and submissive birds depending on the pairing (Dennis, 2009). Environmental factors can act on these genetic predispositions to impact behavioral responses under stressful conditions. The combined effects of environment and genetics mediated through endocrinological and neurological methods suggest that fearful and aggressive behaviors resulting from stressful conditions can be modified through behavioral programming via monoamine levels, including NE, which has neurogenic properties (Jhaveri et al., 2010). Thus, early environmental and epigenetic factors related to adrenal hormone fluctuation can be important considerations in poultry management, particularly in breeding operations.

While less epigenetic research has been conducted in avian models, many mammalian models have shown that maternal stress can significantly impact stress-coping abilities in developed offspring, leading to alterations in hypothalamic-pituitary-adrenal (HPA) axis formation and mood-associated behaviors (Frodl and O'Keane, 2013). The fetal origin hypothesis suggests that the prenatal environment, including physical and psychological maternal stressors, affects long-term behaviors and morphology (Kinsella and Monk, 2009). Multiple human studies have provided evidence to support the fetal origin

hypothesis, associating women who have reported stressful pregnancies with a higher incidence of PTSD, anxiety, and depressive disorders in their young adult children (Watson et al., 1999; Brown et al., 2000; Talge et al., 2007; Kinsella and Monk, 2009). Depression and ADHD related to maternal anxiety and early life stress have been linked to HPA dysregulation and fluctuations in maternal uterine arterial flow leading to inconsistent intrauterine environments (Shnider et al., 1979; Van den Bergh et al., 2008). Numerous rodent and primate studies have also shown that maternal stress can increase anxiety and depressive behaviors in animals (Clarke et al., 1994). Offspring of stressed murine dams demonstrate increased anhedonic states and learned helplessness with decreased coping abilities in novel environment and fear-induction behavioral tests compared to offspring of unstressed dams (Thompson, 1957; Fride et al., 1986; Fride and Weinstock, 1988; Alonso et al., 1991; Vallee et al., 1997; Secoli and Teixeira, 1998; Weinstock, 2002; Morley-Fletcher et al., 2003; Green et al., 2011).

In avian species, incubation and maternal stresses play a significant role in embryonic and post-hatch development in terms of offspring behaviors, physical presentation, and catecholamine and hormone concentrations (Hayward and Wingfield, 2004). Stresses during incubation including asphyxiation, handling, and rapid and prolonged cooling result in an increase in NE, EP, and DA in the chicken egg at ED14 (Epple et al., 1992). Analysis of plasma and allantoic levels of catecholamines suggest that free NE, EP, and DA are produced in embryonic plasma in response to stress and are then transported to the allantois for degradation (Epple et al., 1992; Epple et al., 1997). As a model for elevated catecholamines resulting from maternal or incubation stress with inadequate degradation *in ovo*, injection of 5 μL of 0.03M NE into bobwhite quail eggs at ED21 or ED22 of a 23 day incubation period led to a decreased ability in the post-hatch chicks to recognize a familiar maternal call. Chicks also demonstrated increased embryonic heart rates and temporarily increased post-hatch heart rates due to increased arousal. Results show that elevated levels of embryonic NE late in

development have a significant effect on avian cognition and physiology (Markham et al., 2006). Elevated levels of maternal corticosterone achieved via applied stresses or implants under the skin of the flank result in higher yolk corticosterone concentrations (Hayward and Wingfield, 2004; Saino et al., 2005; Love et al., 2008; Schoech et al., 2011). Physiological parameters such as hatchability rate, body weight, and plumage development are impaired in wild songbirds following elevated yolk corticosterone levels resulting from maternal stress (Saino et al., 2005; Love et al., 2008). In Japanese quail, adult offspring of corticosterone-implanted mothers demonstrate increased HPA axis-related activity in response to handling stresses compared to offspring of non-implanted birds(Hayward and Wingfield, 2004).

There is a vital need to better understand and improve stress-coping mechanisms in avian species given the great impact of fearful and aggressive behaviors on industry profit and bird well-being. Studies of the effects of maternal and incubation stress via increased embryonic monoamines can provide insight into behavioral programming. By utilizing elevated NE concentrations *in ovo* as a model for maternal and incubation stress, we can isolate the effects of catecholamine increase on development and long-term survival-related fear and aggression behaviors throughout offspring growth.

Methods

Treatment

Japanese quail eggs were collected and stored at 11°C three wks prior to the start of incubation. In order to impact early innervation and brain development, fertilized eggs at ED1 were injected with 10 µl of 0.05M ("high dose", n = 130) or 0.01M ("low dose", n = 130) treatment concentrations of L- norepinephrine bitartrate salt monohydrate thoroughly dissolved in sterile saline the day of the injection or 10 µl of sterile saline (n = 130). Concentrations of norepinephrine were chosen and adjusted based on prior literature values

(Markham et al., 2006). Treatments were injected into the albumin using ½ cc tuberculin syringes with 27 gauge needles. Treatment eggs were incubated along with intact controls (n = 80). The procedure was repeated over the course of replicates in time.

Husbandry

After injection on ED1, all eggs were incubated at 99.5°C and 70% relative humidity in a GQF 3258 Digital Control Sportsman® incubator and hatcher for a 16-21 day incubation period. Chicks that pipped but could not emerge from the eggs on their own and chicks that hatched but exhibited deformities interfering with normal functioning were immediately euthanized by cervical dislocation. Within 24 hrs of hatching, healthy, dry chicks were moved to brooding cages set to 95°F and placed on a 24 hr light cycle within treatment groups (high dose, low dose, saline, and intact) of up to 20 individuals. Water was provided in inverted gravity water cups, and Mazuri® Gamebird Starter feed was placed on brown cage paper for the chicks to access *ad libitum* until the chicks were large enough to eat from metal dishes. Once all the chicks for the replicate were hatched, the quail were sorted into treatment groups containing 5-6 individuals per cage.

Each wk, the temperature in the cages was reduced by 5°F until the cages were set to 75-80°F according to standard husbandry operating procedures. After 4 wks of age, the quail were moved in their respective treatment groups from the brooder cages to breeding batteries at 70-75°F and placed on a 12 hr light/dark cycle. The birds received Mazuri® Gamebird Maintenance feed and water *ad libitum*, transitioning from the use of metal dishes to troughs and from inverted gravity water cups to automatic drinkers. Once the birds reached sexual maturity at 6 wks of age, sex ratios were determined and two males from each cage were sampled for physiological data. The females were reorganized by treatment groups into cages of 2-3 non-familiar individuals for further behavioral analysis until the final physiological

sample collection at 11 wks of age to collect organ weights, cecal and intestinal content, and intestinal and brain tissues.

Behavioral Tests

Memory Maze:

A T-maze was used to test the memory of the quail based on perceptual and visual cues at wks 4 and 8. Small dishes of mealworms were placed in the cages for two days prior to training in order for the birds to recognize the mealworms as a reward. Following exposure to the mealworms in the home cage environment, a naïve bird from each cage was selected for memory training in a T-shaped maze. A metal dish was placed in each arm equidistant from the T junction, and one of the dishes was chosen at random to contain mealworms throughout the training and testing for each test subject (Figure 1). In three consecutive days of training, each test subject was placed in the starter box of the T-maze and given 5 minutes to find the dish of mealworms with a few mealworms placed on the ground outside of the correct dish as an additional visual cue on each training day. On the first day of training, if the bird failed to explore both arms of the maze within 5 minutes, the researcher guided the bird to each dish then left the room for another 5 minutes for the bird to explore the maze on its own. For the second and third days of training, the bird was given 5 minutes to choose the arm containing the mealworms. Again, if the bird did not move towards the correct arm after 5 minutes, the researcher guided the bird towards the correct arm then gave the bird an additional 5 minutes to orient itself to the correct arm of the maze.

After three days of training, the birds were given a rest day before testing on the fifth day. On the test day, each bird was placed in the starter box and given 5 minutes to select the arm containing the dish with the mealworms versus the empty dish. No mealworms were placed outside of the dish in order to ensure that the bird received no leading visual cues. Video recording took place over the course of 5 minutes. The latency to move down either

arm, the latency to move down the correct arm, and whether or not the bird chose the correct arm on the first try were analyzed to evaluate memory skills. Moving down either arm was defined as the bird decisively walking at least one-third of the way down the arm. If the bird moved down the correct arm but did not eat the mealworms during the test or within 5 minutes after recording for the test had ended or did not eat the mealworms at least two of the testing days, it was eliminated from the final analysis.

Figure 1. T-maze for memory assessment.

Tonic Immobility:

Individual responses to fear were assessed using the Tonic Immobility (TI) test. A naïve bird from each cage was selected at wks 2, 5, and 8 for testing. In a separate room with a sole observer, each randomly selected bird was placed on its back and restrained across the sternum with its eyes shielded for 10 seconds at a time in a V-shaped cradle draped in a lab pad (Figure 2). The number of attempts needed to induce TI after 10 seconds of sternal restraint and the duration of TI was measured for up to five attempts with the bird remaining in the cradle until it stood up by itself or for a total of 8 minutes.

Figure 2. Tonic Immobility (TI) cradle.

Isolation Test:

Response to isolation was assessed at wks 2, 5, and 8 using naïve birds. A subject from each cage was placed alone in a 3 ft x 3 ft square lined with brown cage paper (Figure 3). The quail was monitored via video recording systems over the course of one minute for latency until the first movement, the number of times the bird defecated, time spent in a stationary versus ambulatory posture and the number of bouts of stationary and ambulatory posturing, and time spent performing a number of behaviors including crouching, remaining erect while vigilant, walking, foraging, exploring, or jumping and attempting to fly (see Appendix I for ethogram). Data were compared between treatments to determine respective strategies of coping with isolation stress.

Figure 3. Open field isolation arena for isolation fear assessment

Home Cage Focal Observations:

Home cage focal observations were used to assess social and aggressive behaviors twenty-four and thirty-six hrs after rehoming following the wk 6 sampling and reorganization of females into cages of two birds as well as at wk 11. Gentle and severe feather pecking, aggressive pecking, play, mounting, and deep-throated call posture frequencies were monitored via an 8-channel digital video recording system (see Appendix I for ethogram). Behavior was recorded for 30 minutes in the morning after the lights in the room were turned on and for 30 minutes in the evening before the lights were turned off when the birds were most active.

Novel Conspecific:

At wk 10, novel groups of two birds from the same treatment but different cage groups were formed temporarily for 20 minute time periods. Reactions towards the novel conspecific were video recorded and analyzed for frequencies of pecks directed at the conspecific and escape attempts. The frequencies exhibited by each treatment were compared

to determine levels of aggression and strategies of coping and establishing pecking order in response to social stress.

Statistical Analysis

Results were checked for normalcy and transformed using a logarithmic or square root function when necessary. If a transformation resulted in a normal distribution, data were analyzed for main effects of treatment and wk as well as interactions between treatment, wk, and replicate using a mixed-model procedure for an Analysis of Variance (ANOVA) in SAS Studio (SAS, Cary, NC). A Sidak correction was performed on P-values produced by differences of least squares means comparisons to identify specific treatment differences within the statistical model. If the data could not be transformed to fit a normal distribution, the sets were analyzed in SAS according to a Chi square and generalized linear model procedure. For both ANOVA and Chi square statistics, P-values less than or equal to 0.05 were accepted as significantly different and P-values greater than 0.05 and less than 0.100 were accepted as a statistical trend.

Results

Memory

Performance results in the memory maze test varied depending on the data sets incorporated into the final analysis. When birds that did not eat the mealworms on testing day during the test or in 5 minutes following the test were included in the data analysis, there were no differences in the time it took to choose the first arm ($P = 0.9186$, $F_{3, 47} = 0.17$), the time it took to choose the correct arm ($P = 0.9134$, $F_{3, 40} = 0.17$), or whether the correct arm was chosen on the first attempt ($P = 0.3279$, $F_{3, 47} = 1.18$). We conducted a second data analysis excluding birds that did not eat the mealworms on the testing day with different results. N-values were lower for birds that choose the correct arm compared to birds that

choose either arm as some individuals failed to choose correctly throughout the period given to complete the maze (Table 2).

Table 2. N-values for each memory assessment including only birds that ate the mealworms on the testing day. N-values refer to an individual bird.

Treatment	Latency to First Arm		Latency to Correct Arm		Percentage Choosing Correctly	
	Wk 4	Wk 8	Wk 4	Wk 8	Wk 4	Wk 8
High	6	6	6	5	6	6
Low	7	6	6	6	7	6
Saline	7	4	7	4	7	4
Intact	4	5	3	5	4	5

When birds that did not eat the mealworms on the testing day were excluded from the statistical analysis, there were main effects of treatment in the time it took to choose the first arm ($P = 0.03$, $F_{3, 37} = 3.24$). High dose birds performed variably on the memory test but demonstrated the greatest average latency to move down the first arm followed by intact birds, low dose birds, and saline birds for both wk 4 and wk 8 (Figure 4). For wk 4, the average latency to move down the first arm was 45 ± 37 s for high, 11 ± 4 s for low dose, 6 ± 2 s for saline, and 36 ± 9 s for intact birds. For wk 8, the average latency to move down the first arm was 41 ± 28 s for high dose, 8 ± 2 s for low dose, 4 ± 1 s for saline, and 22 ± 1 s for intact birds. Saline birds demonstrated significantly lower latencies than high dose ($P = 0.0216$) and intact birds ($P = 0.0095$) birds and low dose birds tended to exhibit lower latencies than intact birds ($P = 0.0734$).

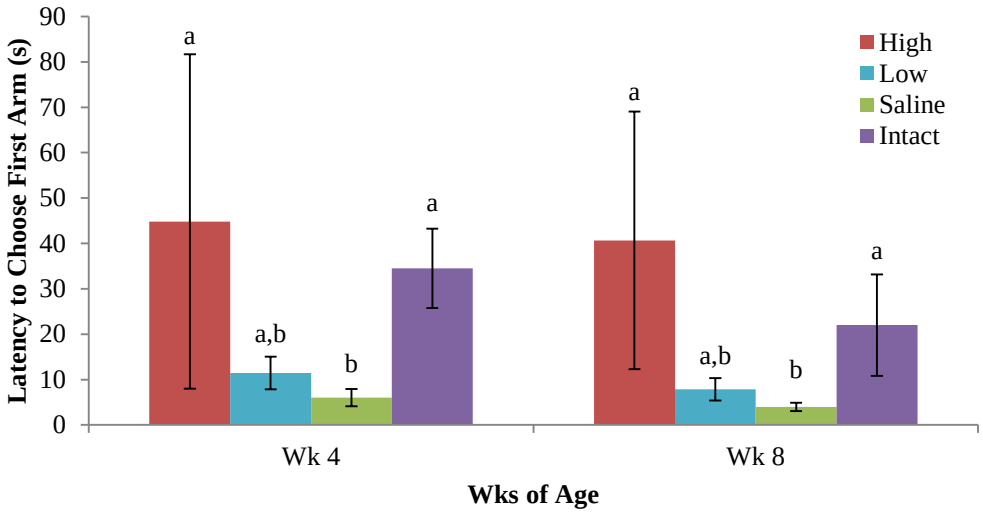

Figure 4. The latency to walk down the first arm chosen in the T-maze. Different letters denote a significant difference between treatments ($P < 0.05$).

There were main treatment tendencies in the time it took to choose the correct arm ($P = 0.06$, $F_{3, 34} = 2.75$) excluding birds that did not eat the mealworms on the testing day. Intact birds took the most time to choose the correct arm followed by high dose birds. Low dose birds took more time to choose the correct arm at wk 4 than saline birds, but saline birds took more time than low dose birds at wk 8 (Figure 5). At wk 4, there was a latency of 47 ± 36s for high dose, 32 ± 14 s for low dose, 13 ± 3 s for saline, and 56 ± 26 s for intact birds. At wk 8, there was a latency of 22 ± 9 s for high dose, 8 ± 2 s for low dose, 10 ± 13 s for saline, and 55 ± 19 s for intact birds. Intact birds demonstrated greater latencies than low dose ($P = 0.0283$) and saline ($P = 0.0104$) birds.

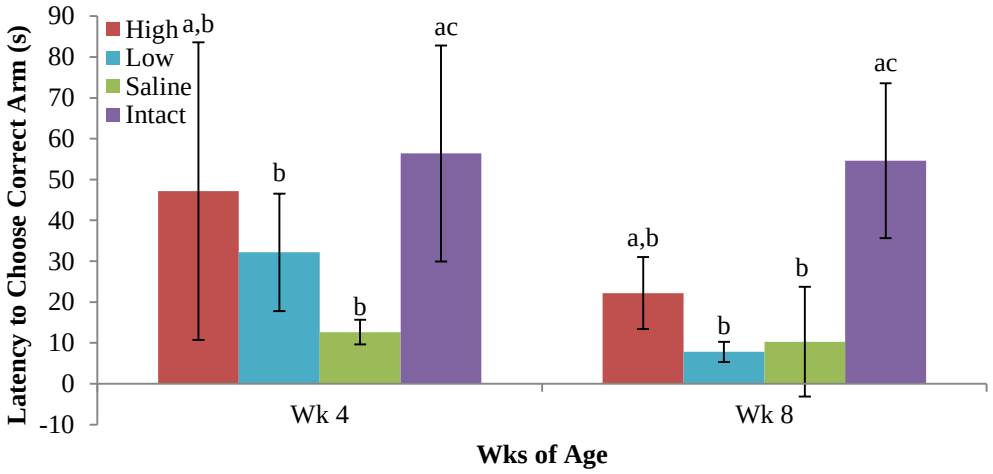

Figure 5. The latency to walk down the correct arm in the T-maze. Different letters denote a significant difference between treatments ($P < 0.05$).

There were no differences in treatment-age interactions in whether the correct arm was chosen on the first attempt ($P = 0.23$, $F_{3, 37} = 1.5$) excluding birds that did not eat the mealworms on the testing day. The percentage of birds choosing correctly on the first attempt was variable between treatments and wks (Figure 6). At wk 4, 83% of high dose, 57% of low dose, 71% of saline, and 50% of intact birds choose the correct arm on the first attempt. At wk 8, 50% of high dose, 100% of low dose, 75% of saline, and 40% of intact birds choose the arm with mealworms on the first attempt.

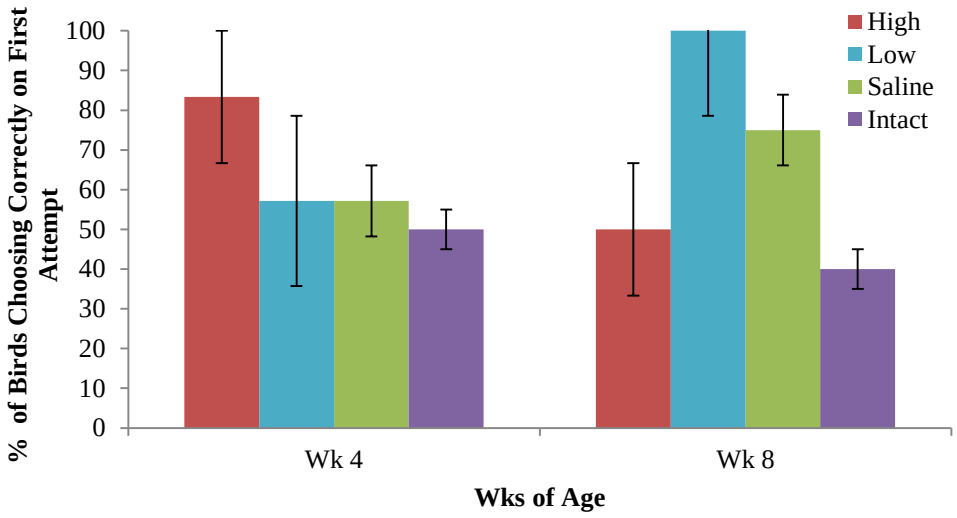

Figure 6. The percentage of birds that chose the correct arm on the first attempt walking down an arm of the T-maze. There were no significant differences between treatments ($P > 0.05$).

Tonic Immobility

Higher TI durations indicate that a bird is experiencing a greater level of predator-based fear while the number of inductions assesses fear thresholds. In our study, not all individual birds attained TI within five induction attempts resulting in different n-values between TI duration and number of inductions (Table 3). Data were analyzed with and without the intact treatment due to a low n-value for intact birds that exhibited TI within five induction attempts.

Table 3. N-values for each TI assessment. N-values refer to an individual bird.

Treatment	**TI Duration**			**TI Inductions**		
	Wk 2	Wk 5	Wk 9	Wk 2	Wk 5	Wk 9
High	6	6	6	7	7	8
Low	6	7	8	9	9	9
Saline	8	8	9	9	9	10
Intact	3	4	5	5	5	5

Tonic immobility duration varied between treatments with low dose birds initially exhibiting the highest duration, or fear levels, as chicks. Differences between the treatments

minimized over time as TI duration for the low dose birds decreased (Figure 7). Overall, there was a main treatment tendency in TI duration ($P = 0.0733$, $F_{3, 35} = 2.82$) when data were analyzed excluding the intact treatment and a main age tendency ($P = 0.0713$, $F_{2, 64} = 2.74$) when data were analyzed including the intact treatment. At wk 2, TI durations were 76 ± 26 s for high dose, 221 ± 57 s for low dose, 121 ± 34 s for saline, and 154 ± 21 s for intact birds with low dose birds tending to exhibit a higher TI duration than high dose birds when intact data were excluded ($P = 0.0720$). At wk 5, TI durations were 71 ± 21 s for high dose, 160 ± 55 s for low dose, 111 ± 44 s for saline, and 57 ± 16 s for intact birds. At wk 9, TI durations were 85 ± 31 s for high dose, 116 ± 46 s for low dose, 95 ± 48 s for saline, and 174 ± 93 s for intact birds. The intact birds tended to exhibit a lower duration at wk 5 than wk 2 ($P = 0.0882$).

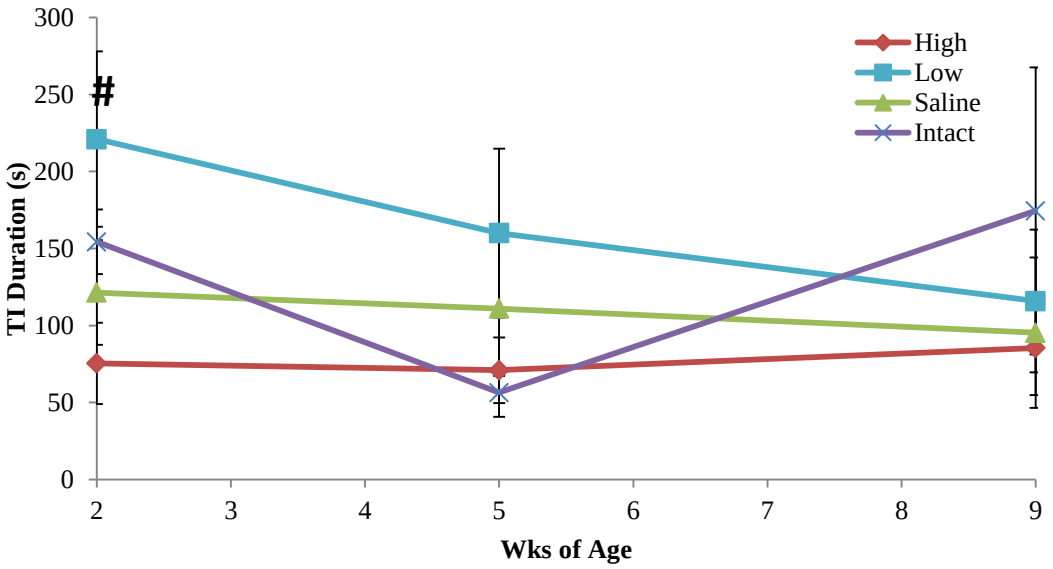

Figure 7. The amount of time birds spent in tonic immobility. # denotes a trend ($0.05 < P < 0.10$).

A low number of TI inductions indicate that a bird is more fearful. All treatments exhibited similar numbers of average TI inductions but slightly different patterns over time (Figure 8). There were no differences in treatment-age interactions for TI inductions

excluding the intact treatment (P = 0.8291, $F_{2,44}$ = 0.19) or including the intact treatment (P = 0.3559, $F_{6,78}$ = 1.33). At wk 2, there were 2.4 ± 0.7 inductions for high dose, 2.9 ± 0.6 inductions for low dose, 2.9 ± 0.6 inductions for saline, and with 3.6 ± 0.6 inductions for intact birds. At wk 5, there were 2.4 ± 0.5 inductions for high dose, 2, 2.7 ± 0.5 inductions for low dose, 3.0 ± 0.6 inductions for saline, and 3.8 ± 0.6 inductions for intact birds. At wk 9, there were 2.9 ± 0.7 inductions for high dose, 2.2 ± 0.5 inductions for low dose, 2.7 ± 0.5 inductions for saline, and 1.4 ± 0.4 inductions for intact birds.

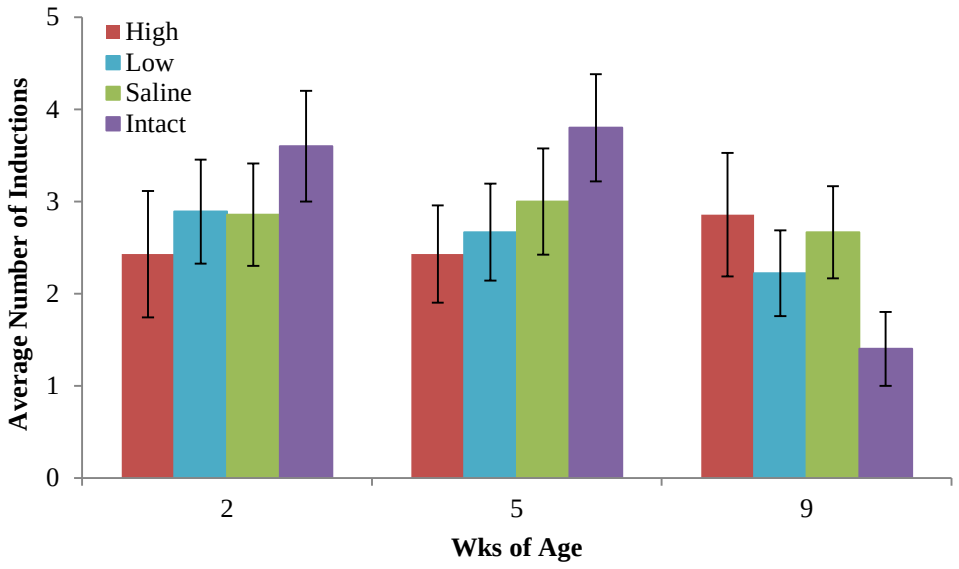

Figure 8. The number of attempts needed to induce tonic immobility up to a maximum of five tries. There were no significant differences between treatments (P > 0.05).

Open Field Isolation Test

Open field isolation test data was extremely variable and required to use of non-parametric statistical analysis. Chi square and generalized linear model procedures were used to determine significance. N-values remained the same for wks 2 and 5 but changed following rehoming at sexual maturity (Table 4).

Table 4. N-values for the open field isolation test. N-values refer to an individual bird.

Treatment	Wk 2	Wk 5	Wk 8
High	7	7	7
Low	9	9	10
Saline	9	9	12
Intact	5	5	4

High dose birds exhibited an increase in latency until the first body movement at wk 5, but all other treatments showed a decrease in latency until the first body movement over time with control birds having a greater latency in the later wks (Figure 9). However, there were no overall Chi-square differences between treatments ($P = 0.9779$, $F_{3, 91} = 0.06$). At wk 2, there was a latency of 19 ± 11 s for high dose, 17 ± 7 s for low dose, 16 ± 8 s for saline, and 19 ± 12 s for intact birds. At wk 5, there was a latency of 27 ± 11 s for high dose, 9 ± 6 s for low dose, 14 ± 9 s for saline, and 15 ± 15 s for intact birds. At wk 8, there was a latency of 5 ± 1 s for high dose, 2 ± 0 s for low dose, 3 ± 1 s for saline, and 3 ± 1 s for intact birds.

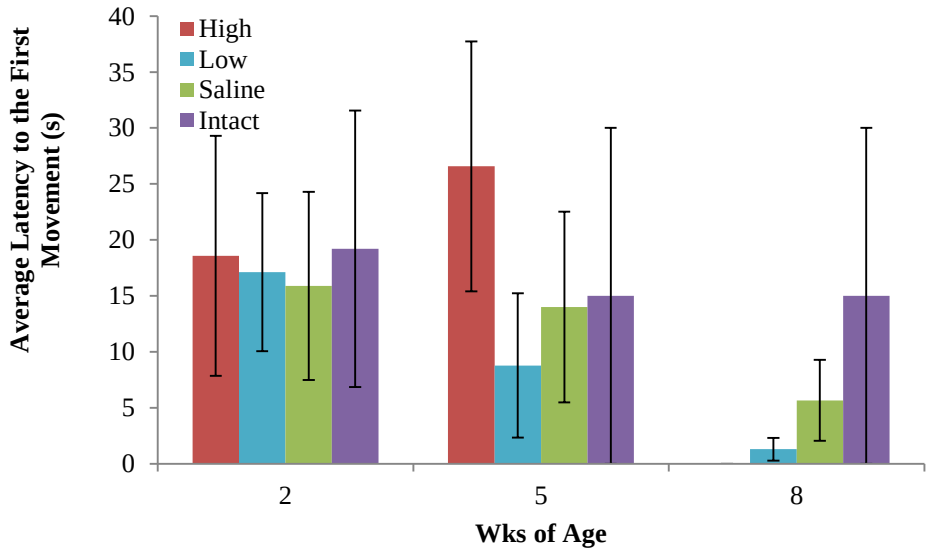

Figure 9. Average latency until the first movement over the course of 1 minute in isolation in an open field arena. There were no Chi square differences between treatments ($P > 0.05$).

High dose birds defecated more than low dose, saline, and intact birds at wk 2 and wk 8 while the other treatments showed less variation depending upon the wk of testing (Figure

10). Overall, there were Chi square trends between treatments (P = 0.0797, $F_{3, 90}$ = 2.36) with high dose birds defecating more. At wk 2, high dose birds defecated an average of 1 ± 1 times, low dose birds defecated an average of less than 1 ± 1 times, saline birds defecated an average of less than 1 ± 1 times, and intact birds defecated in average of less than 1 ± 1 times. At wk 5, high dose birds defecated an average of 1 ± 0 times, low dose birds defecated an average of 1 ± 0 times, saline birds defecated an average of 1 ± 0 times, and intact birds defecated an average of 1 ± 0 times. At wk 8, high dose birds defecated an average of 2 ± 0 times, low dose birds defecated an average of less than 1 ± 1 times, saline birds defecated an average of 1 ± 0 times, and intact birds defecated an average of 1 ± 0 times.

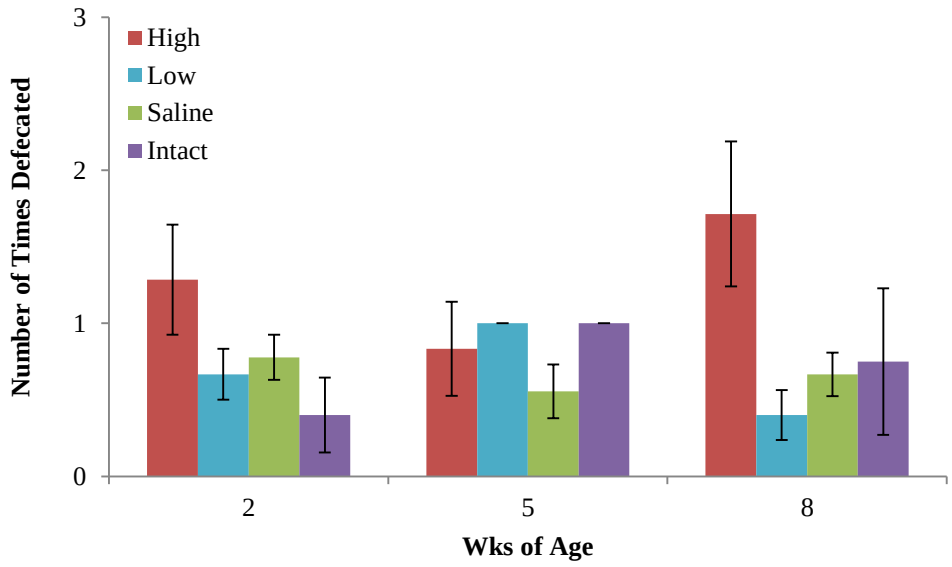

Figure 10. Average number of times defecated over the course of 1 minute in isolation in an open field arena. High dose birds demonstrated a tendency to defecate more than other treatments (0.05 < P < 0.10).

Time allotment of behaviors during the isolation test was variable depending on the treatment and wk. Time spent ambulatory increased in the NE-treated birds over time. High and low dose birds appeared to spend more time ambulatory compared to control birds at wk 2 and wk 8 while control birds spent more time ambulatory at wk 5 (Figure 11). However, there were no overall Chi-square differences between treatments (P = 0.6166, $F_{3, 91}$ = 0.06).

At wk 2, time spent ambultaory was 11 ± 4 s for high dose, 6 ± 2 s for low dose, 3 ± 1 s for saline, and 3 ± 1 s for intact birds. At wk 5, time spent ambultaory was 9 ± 5 s for high dose, 10 ± 3 s for low dose, 16 ± 1 s for saline, and 17 ± 12 s for intact birds. At wk 8, time spent ambulatory was 21 ± 6 s for high dose, 14 ± 6 s for low dose, 13 ± 4 s for saline, and 10 ± 7 s for intact birds.

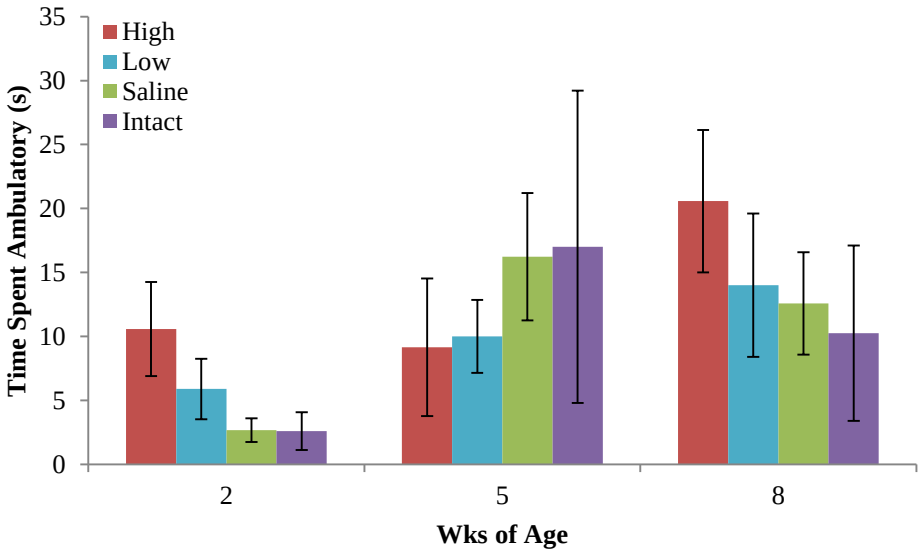

Figure 11. Average time spent ambulatory over the course of 1 minute in isolation in an open field arena. There were no Chi square differences between treatments ($P > 0.05$).

The number of ambulatory bouts was generally consistent between the treatments and the wks although the high dose birds had more bouts of ambulatory behavior at wk 2 and wk 8 (Figure 12). However, there were no overall Chi-square differences between treatments ($P = 0.2162$ $F_{3, 91} = 1.51$). At wk 2, the average number of ambulatory bouts was 3 ± 1 for high dose, 2 ± 1 for low dose, 2 ± 1 for saline, 1 ± 0 for intact birds. At wk 5, the average number of ambulatory bouts was 2 ± 1 for high dose, 2 ± 1 for low dose, 2 ± 0 for saline, and 2 ± 1 for intact birds. At wk 8, the average number of ambulatory bouts was 5 ± 1 for high dose, 2 ± 1 for low dose, 3 ± 1 for saline, and 3 ± 1 for intact birds.

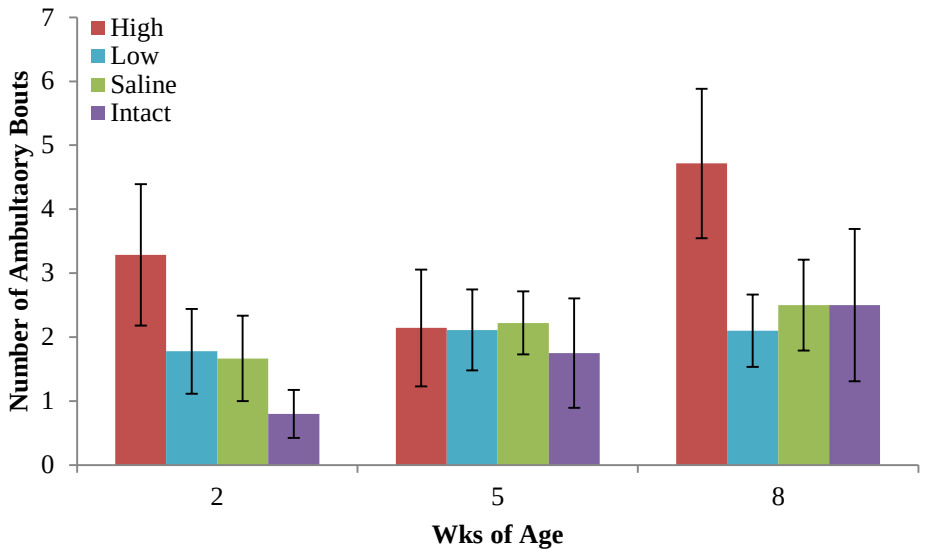

Figure 12. Average number of ambulatory bouts over the course of 1 minute in isolation in an open field arena. There were no Chi square differences between treatments ($P > 0.05$).

High and low dose birds spent less time stationary compared to control birds at wk 2 and wk 8 while control birds spent less time stationary at wk 5. Low dose birds spent less time stationary over time (Figure 13). However, there were no overall Chi-square differences between treatments ($P = 0.6096$, $F_{3, 91} = 0.60$). At wk 2, time spent stationary was 49 ± 4 s for high dose, 54 ± 2 s for low dose, 57 ± 0 s for saline, and 57 ± 1 s for intact birds. At wk 5, time spent stationary was 51 ± 5 s for high dose, 50 ± 3 s for low dose, 44 ± 5 s for saline, and 43 ± 12 s for intact birds. At wk 8, time spent stationary was 39 ± 6 s for high dose, 46 ± 6 s for low dose, 47 ± 4 s for saline, and 50 ± 7 s for intact birds.

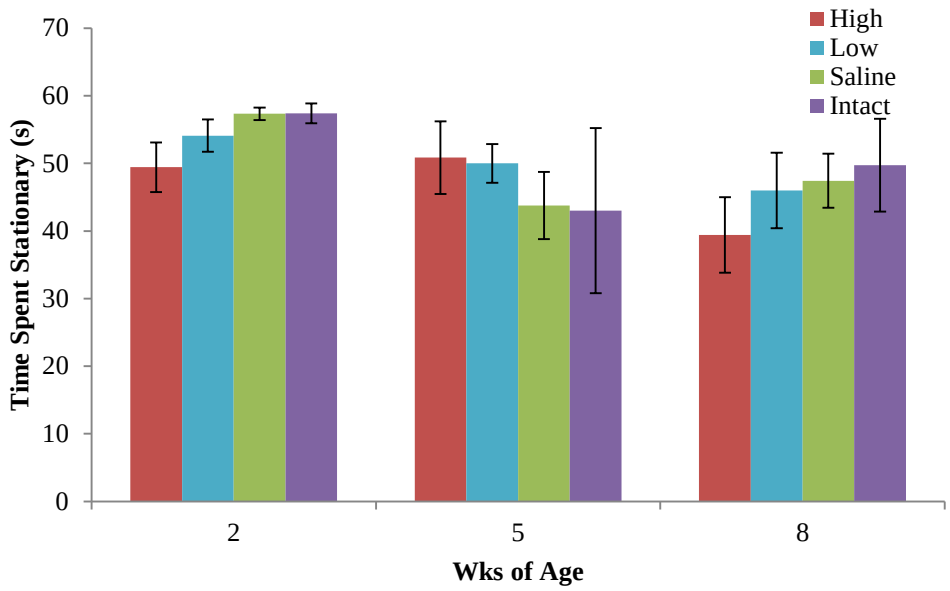

Figure 13. Average time spent stationary over the course of 1 minute in isolation in an open field arena. There were no Chi square differences between treatments ($P > 0.05$).

High dose birds exhibited a greater number of stationary bouts at wk 2 and wk 8 compared to other treatments. Low dose birds exhibited fewer stationary bouts than high dose birds but remained consistent throughout the wks. Saline and intact birds increased in the number of stationary bouts over time, initially showing fewer stationary bouts compared to NE-treated birds then more stationary bouts than low dose birds (Figure 14). Overall, there were Chi square trends between treatments ($P = 0.0896$, $F_{3, 91} = 2.26$) with high dose birds demonstrating more stationary bouts compared to other treatments. At wk 2, the average number of stationary bouts was 4 ± 1 for high dose, 2 ± 1 for low dose, 2 ± 1 for saline, and 1 ± 0 for intact birds. At wk 5, the average number of stationary bouts was 2 ± 1 for high dose, 2 ± 1 for low dose, 3 ± 0 for saline, and 1 ± 1 for intact birds. At wk 8, the average number of stationary bouts was 5 ± 1 for high dose, 2 ± 0 for low dose, 3 ± 1 for saline, and 3 ± 1 for intact birds.

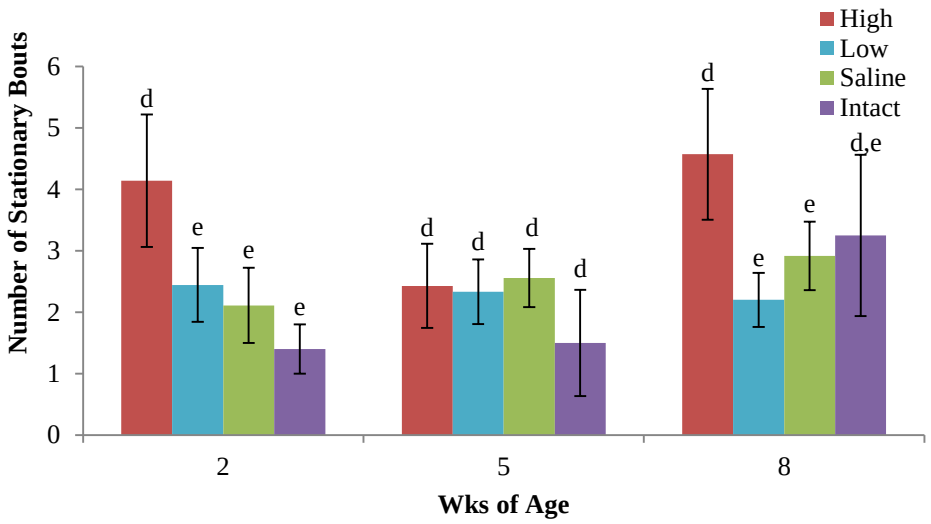

Figure 14. Average number of stationary bouts over the course of 1 minute in isolation in an open field arena. Different letters denote a trend between treatments ($0.05 < P < 0.10$).

Control birds spent more time crouching compared to NE-treated birds in wk 2 and wk 8 with almost no NE-treated birds crouching in wk 8. However, at wk 5, high dose birds spent more time crouching while no intact birds spent time crouching. High and low dose birds spent less time crouching compared to control birds at wk 2 and wk 8 while control birds spent less time crouching at wk 5. Low dose birds spent less time crouching over time (Figure 15). However, there were no overall Chi square differences between treatments ($P = 0.5376$, $F_{3, 91} = 0.72$). At wk 2, time spent crouching was 19 ± 11 s for high dose, 13 ± 7 s for low dose, 40 ± 8 s for saline, and 30 ± 13 s for intact birds. At wk 5, time spent crouching was 33 ± 12 s for high dose, 18 ± 8 s for low dose, 21 ± 8 s for saline, and 0 ± 0 s for intact birds. At wk 8, time spent crouching was 0 ± 0 s for high dose, 0 ± 0 s for low dose, 6 ± 3 s for saline, and 17 ± 14 s for intact birds.

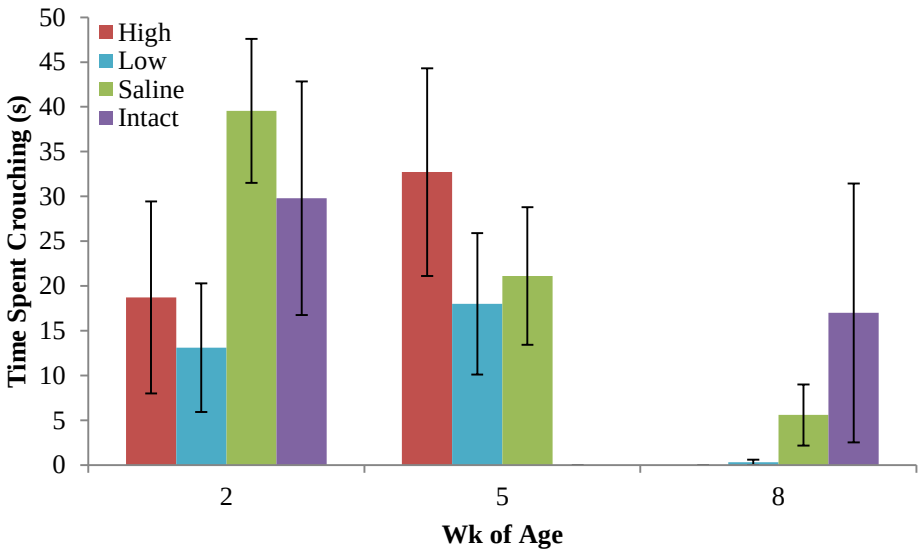

Figure 15. Average time spent crouched over the course of 1 minute in isolation in an open field arena. There were no Chi square differences between treatments ($P > 0.05$).

Low dose birds spent more time erect and vigilant compared to other treatments in each wk with the exception of intact birds at wk 5 (Figure 16). However, there were no overall Chi square differences between treatments ($P = 0.1063$, $F_{3, 91} = 2.11$). At wk 2, time spent erect and vigilant was18 ± 7 s for high dose, 41 ± 8 s for low dose, 12 ± 6 s for saline, and 26 ± 12 s for intact birds. At wk 5, time spent erect and vigilant was18 ± 9 s for high dose, 33 ± 9 s for low dose, 26 ± 8 s for saline, 42 ± 13 s for intact birds. At wk 8, time spent erect and vigilant was 36 ± 7 s for high dose, 41 ± 7 s for low dose, 26 ± 8 s for saline, and 33 ± 12 s for intact birds.

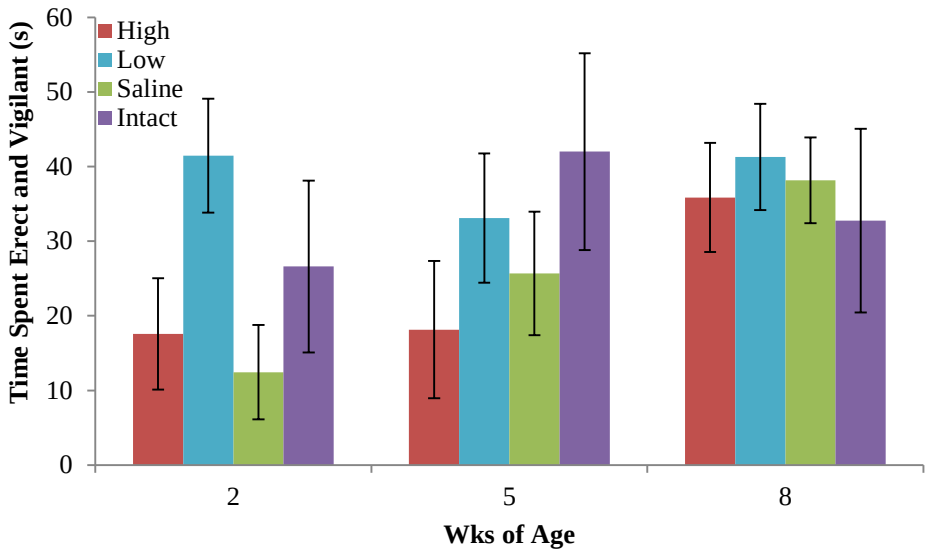

Figure 16. Average time spent erect and vigilant over the course of 1 minute in isolation in an open field arena. There were no Chi square differences between treatments ($P > 0.05$).

Norepinephrine-treated birds spent more time walking compared to control birds at wk 2 and wk 8 while saline birds walked more at wk 5. High dose birds spent more time walking at wk 2 compared to other treatments, but at wk 5 and wk 8, high dose birds spent similar amounts of time walking as low dose birds (Figure 17). There were no overall Chi square differences between treatments ($P = 0.3467$, $F_{3, 91} = 1.11$). At wk 2, time spent walking was 19 ± 8 s for high dose, 5 ± 2 s for low dose, 1 ± 1 s for saline, and 2 ± 2 s for intact birds. At wk 5, time spent walking was 8 ± 5 s for high dose, 9 ± 3 s for low dose, 13 ± 6 s for saline, and 5 ± 2 s for intact birds. At wk 8, time spent walking was 12 ± 3 s for high dose, 13 ± 5 s for low dose, 9 ± 3 s for saline, and 10 ± 7 s for intact birds.

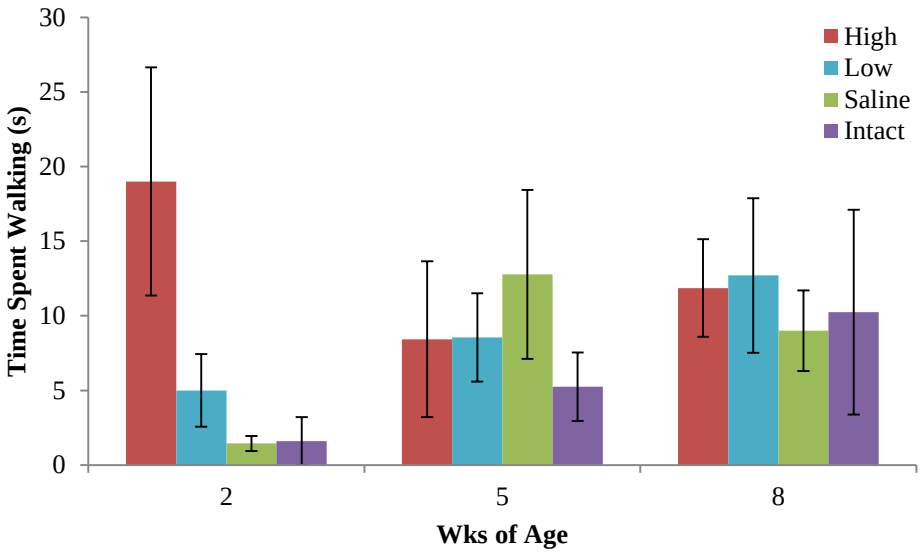

Figure 17. Average time spent walking over the course of 1 minute in isolation in an open field arena. There were no Chi square differences between treatments (P > 0.05).

Foraging behavior was limited during the isolation test but there were Chi square differences between treatments (P = 0.0207, $F_{3, 91} = 3.53$) with saline birds foraging for more time compared to other treatments. At wk 2, saline birds spent an average of less than 1 ± 1 s foraging while no birds from the other spent time foraging. At wk 5, saline birds spent an average of less than 1 ± 1 s foraging. At wk 8, high dose birds spent 3 ± 3 s foraging and saline birds spent 3 ± 2 s foraging while low dose and intact birds did not spend any time foraging.

Exploratory behavior was limited during the isolation test with birds at wk 8 showing the most exploratory behavior. However, there were no overall Chi square differences between treatments (P = 0.6695, $F_{3, 91} = 0.52$). At wk 2, saline birds spent 5 ± 5 s exploring the arena while no birds from the other treatments spent time exploring. At wk 5, high dose birds spent an average of less than 1 ± 1 s exploring and intact birds spent 13 ± 13 s exploring while no low dose birds or saline birds spent time exploring the arena. At wk 8, time spent exploring was 5 ± 4 s for high dose, 1 ± 1 s for low dose, and 4 ± 2 s for saline birds. Intact birds did not spend any time exploring at wk 8.

Jumping and flight attempts were limited during the isolation test with low dose and saline birds exhibiting similar amounts of time performing the behaviors at wk 2, no birds exhibiting the behaviors at wk 5, and NE-treated birds spending more time performing the behaviors at wk 8 compared to control birds. However, there were no overall Chi square differences between treatments ($P = 0.5244$, $F_{3, 91} = 0.75$). At wk 2, both low dose birds and saline birds spent an average of less than 1 ± 1 s jumping and attempting to fly while high dose birds and intact birds did not demonstrate jumping and flight behaviors. At wk 8, average time spent jumping and attempting to fly was 1 ± 1 s for high dose, less than 1 ± 1 s for low dose, and less than 1 ± 1 s for saline birds. Intact birds did not perform these behaviors.

Norepinephrine-treated birds spent more time performing other behaviors including scratching at the floor, flapping wings, and vocalizing compared to control birds. However, there were no overall Chi square differences between treatments ($P = 0.3299$, $F_{3, 91} = 1.15$). At wk 2, time spent performing other behaviors was 5 ± 3 s for high dose, less than 1 ± 1 s for low dose, 1 ± 1 s for saline, and 2 ± 2 s for intact birds. At wk 5, time spent performing other behaviors was 1 ± 1 s for high dose and less than 1 ± 1 s for low dose birds. Saline and intact birds did not perform other behaviors. At wk 8, time spent performing other behaviors was 3 ± 3 s for high dose and 4 ± 4 s for low dose birds. Saline and intact birds did not perform other behaviors.

Home Cage Focal Observations

Social, aggressive, and reproductive behaviors were highly variable between the treatments and the sampling times (Table 5) for thehome cage focal observations and required to use of non-parametric statistical analysis. Chi square and generalized linear model procedures were used to determine significance. Twenty-four hrs after rehoming at wk 6 during an evening observation, high dose birds demonstrated greater levels

of gentle feather pecking, low dose birds and intact birds demonstrated similar levels of gentle feather pecking, and saline birds demonstrated lesser levels of gentle feather pecking although there were no Chi square differences between treatments ($P = 0.2140$, $F_{3, 31} = 1.58$). No birds exhibited play behavior. Low dose birds displayed some severe pecking and mounting behavior while other treatments did not with no Chi square differences in either behavior ($P = 0.5915$, $F_{3, 31} = 0.61$ and $P = 0.5915$, $F_{3, 31} = 0.61$, respectively). No birds made escape attempts or assumed a deep-throated call posture.

Thirty-six hrs after rehoming at wk 6 during a morning observation, high birds demonstrated higher levels of gentle feather pecking, control birds demonstrated similar levels of gentle feather pecking, and low dose birds demonstrated low levels of gentle feather pecking although there were no Chi square differences between treatments ($P = 0.9415$, $F_{3, 29} = 0.12$). No birds exhibited play behavior. High dose birds displayed severe pecking behavior while other treatments did not with no Chi square differences between treatments ($P = 0.1718$, $F_{3, 29} = 1.81$). No birds exhibited mounting behavior. High dose birds made some escape attempts. Low dose birds and intact birds made fewer escape attempts, but there were no Chi square differences between treatments ($P = 0.2021$, $F_{3, 29} = 1.64$). No birds assumed the deep-throated call posture.

During the AM observation at wk 11, intact birds demonstrated high levels of gentle feather pecking followed by low dose and high dose birds while saline birds did not exhibit gentle feather pecking. There were Chi square trends between treatments ($P = 0.0576$, $F_{3, 28} = 3.05$) with intact birds gently feather pecking more than saline and high dose birds. No birds exhibited severe pecking or play behavior. Saline birds displayed some mounting behavior while other treatments did not with no Chi square differences between treatments ($P = 0.1870$, $F_{3, 28} = 1.72$). No birds exhibited escape attempts or deep-throated call postures.

During the PM observation at wk 11, high dose birds demonstrated high levels of gentle feather pecking, control birds demonstrated similar levels of gentle feather pecking,

and low dose birds demonstrated lower levels of gentle feather pecking with no Chi square differences between treatments (P = 0.5499, $F_{3, 34}$ = 0.68). No birds exhibited play behavior. High dose birds displayed some severe pecking behavior while other treatments did not with no Chi square differences between treatments (P = 0.2615, $F_{3, 34}$ = 1.38). Intact birds demonstrated some mounting behavior and escape attempts while other treatments did not with no Chi square differences between treatments in either behavior (P = 0.1116, $F_{3, 34}$ = 2.21 and P = 0.1116, $F_{3, 34}$ = 2.21, respectively). Low dose birds assumed deep-throated call postures while other birds did not with no Chi square differences between treatments (P = 0.5355; $F_{3, 34}$ = 0.71).

Table 5. The number of behaviors performed per bird within a half hr of the lights coming on in the morning and going off at night on a 12 hr lighting schedule at wk 6, 24 hrs (PM) and 36 hrs (AM) following rehoming, and at wk 11

		High		**Low**		**Saline**		**Intact**	
	Time	Behavior/ Bird	N	Behavior/ Bird	N	Behavior/ Bird	N	Behavior/ Bird	N
Gentle Feather Pecking	Wk 6 - 24 hrs	0.72 ± 0.34	6	0.41 ± 0.20	11	0.10 ± 0.07	10	0.47 ± 0.29	5
	Wk 6 - 36 hrs	0.42 ± 0.42	6	0.11 ± 0.07	11	0.25 ± 0.13	10	0.20 ± 0.20	5
	Wk 11 AM	0.07 ± 0.07	7	0.17 ± 0.10	11	0.00 ± 0.00	12	0.43 ± 0.19	5
	Wk 11 PM	0.48 ± 0.42	7	0.17 ± 0.10	11	0.38 ± 0.22	12	0.33 ± 0.09	5
Play	Wk 6 - 24 hrs	0.00 ± 0.00	6	0.00 ± 0.00	11	0.00 ± 0.00	10	0.00 ± 0.00	5
	Wk 6 - 36 hrs	0.00 ± 0.00	6	0.00 ± 0.00	11	0.00 ± 0.00	10	0.00 ± 0.00	5
	Wk 11 AM	0.00 ± 0.00	7	0.00 ± 0.00	11	0.00 ± 0.00	12	0.00 ± 0.00	5
	Wk 11 PM	0.00 ± 0.00	7	0.00 ± 0.00	11	0.00 ± 0.00	12	0.00 ± 0.00	5
Severe Pecking	Wk 6 - 24 hrs	0.00 ± 0.00	6	0.05 ± 0.05	11	0.00 ± 0.00	10	0.00 ± 0.00	5
	Wk 6 - 36 hrs	0.08 ± 0.20	6	0.00 ± 0.00	11	0.00 ± 0.00	10	0.00 ± 0.00	5
	Wk 11 AM	0.00 ± 0.00	7	0.00 ± 0.00	11	0.00 ± 0.00	12	0.00 ± 0.00	5
	Wk 11 PM	0.07 ± 0.07	7	0.00 ± 0.00	11	0.00 ± 0.00	12	0.00 ± 0.00	5
Mounting	Wk 6 - 24 hrs	0.00 ± 0.00	6	0.05 ± 0.05	11	0.00 ± 0.00	10	0.00 ± 0.00	5
	Wk 6 - 36 hrs	0.00 ± 0.00	6	0.00 ± 0.00	11	0.00 ± 0.00	10	0.00 ± 0.00	5
	Wk 11 AM	0.00 ± 0.00	7	0.00 ± 0.00	11	0.00 ± 0.00	12	0.06 ± 0.06	5
	Wk 11 PM	0.00 ± 0.00	7	0.00 ± 0.00	11	0.00 ± 0.00	12	0.13 ± 0.13	5
Escape Attempts	Wk 6 - 24 hrs	0.00 ± 0.00	6	0.00 ± 0.00	11	0.00 ± 0.00	10	0.00 ± 0.00	5
	Wk 6 - 36 hrs	0.14 ± 0.09	6	0.05 ± 0.05	11	0.00 ± 0.00	10	0.07 ± 0.07	5
	Wk 11 AM	0.00 ± 0.00	7	0.00 ± 0.00	11	0.00 ± 0.00	12	0.00 ± 0.00	5
	Wk 11 PM	0.00 ± 0.00	7	0.00 ± 0.00	11	0.00 ± 0.00	12	0.06 ± 0.06	5
Deep-throated Call Posture	Wk 6 - 24 hrs	0.00 ± 0.00	6	0.00 ± 0.00	11	0.00 ± 0.00	10	0.00 ± 0.00	5
	Wk 6 - 36 hrs	0.00 ± 0.00	6	0.00 ± 0.00	11	0.00 ± 0.00	10	0.00 ± 0.00	5
	Wk 11 AM	0.00 ± 0.00	7	0.00 ± 0.00	11	0.00 ± 0.00	12	0.00 ± 0.00	5
	Wk 11 PM	0.00 ± 0.00	7	0.05 ± 0.05	11	0.00 ± 0.00	12	0.00 ± 0.00	5

Novel Conspecific Test

Birds placed with a novel conspecific of the same treatment at wk 11 for 20 minutes exhibited pecking frequencies proportional to NE dose (Figure 18) with main effects of treatment ($P = 0.0386$, $F_{2, 14} = 4.14$). High dose pairs (N = 4) had a pecking mean of 23 ± 4 pecks per cage, low dose pairs (N = 6) had a mean of 14 ± 3 pecks per cage, and saline pairs (N = 7) had a mean of 8 ± 4 pecks per cage. High dose birds pecked at a conspecific significantly more than saline birds ($P = 0.0440$).

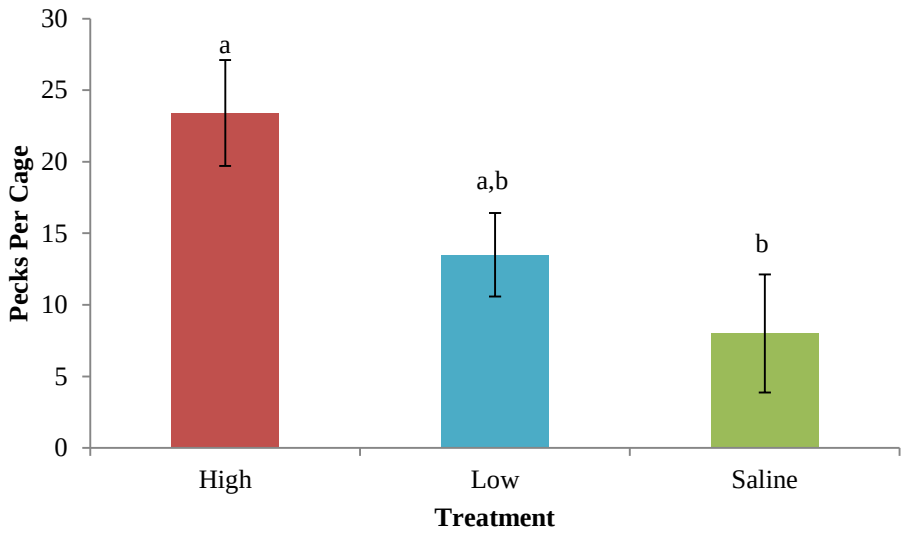

Figure 18. Pecking between novel conspecifics of the same treatment when placed together for 20 minutes at wk 11. Different letters denote a significant difference between treatments ($P < 0.05$).

Low dose birds demonstrated more escape attempts than the other treatments when placed with a novel conspecific of the same treatment (Figure 19). There was a main treatment tendency ($P = 0.0665$, $F_{2, 14} = 3.31$) in the number of escape attempts per cage over a 20 minute span. High dose pairs (N = 4) had a mean of 6 ± 3 for escape attempts per cage, low dose pairs (N = 6) had a mean of 14 ± 3 escape attempts per cage, and saline pairs (N = 7) had a mean of 6 ± 4 escape attempts per cage.

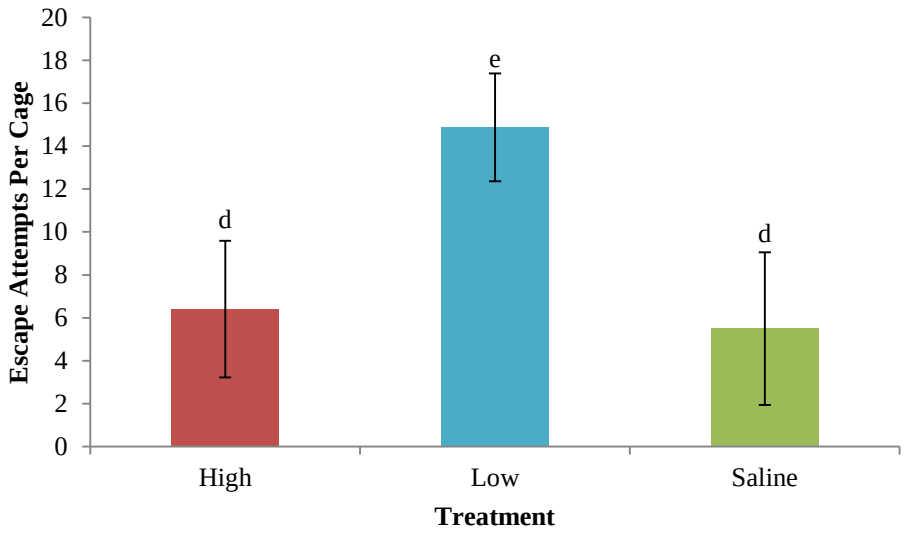

Figure 19. Escape attempts from novel conspecifics of the same treatment when placed together for 20 minutes at wk 11. Different letters denote a trend between treatments ($0.05 < P < 0.10$).

Discussion

This study assessed the effects of high and low levels of embryonic NE on cognitive, fearful, and aggressive behaviors throughout stages of post-hatch development. Our data provide evidence that elevated embryonic NE affects cognitive processes and stress-coping mechanisms in a dose- and age-dependent manner. In the memory test, high dose birds demonstrated a greater latency to choose the first arm and the correct arm compared to saline birds while low dose birds demonstrated lower latencies compared to intact birds. High dose birds chose the correct arm on the first attempt more frequently as juveniles than adults while low dose birds chose the correct arm more frequently as adults than juveniles. In a TI test, low dose chicks tended to exhibit longer TI durations than high dose and saline chicks. However, there were no treatment differences in TI duration in juvenile or mature birds or in the number of inductions needed to establish TI at any age. These data indicate that while the threshold needed to induce fear was similar between treatments, there were dose- and age-dependent differences in how long an animal remained in a fearful state. Similarly, in an open field isolation test, high and low dose birds coped with predatory and isolation stress

differently and exhibited more fearful behaviors than control birds. High dose birds tended to transition between moving and remaining sedentary more and foraged less than control birds while low dose birds remained more sedentary. High dose and low dose birds also defecated more than control birds. While we did not see treatment differences in social, aggressive, and reproductive behaviors 24 and 36 hours following rehoming at wk 6, by wk 11 intact birds tended to gently feather peck familiar conspecifics more than high dose birds. In comparison, high dose birds pecked novel conspecifics more frequently demonstrating a confrontational strategy while low dose birds tended to exhibit greater number of escape attempts demonstrating an evasion-based strategy or more variability in hierarchical behaviors in response to social stress. Overall, we can conclude that elevated embryonic NE differentially impacts behavioral responses related to cognition, fear, aggression, and sociality throughout post-hatch growth based on *in ovo* NE concentrations at the start of embryogenesis.

Data from prior research have repeatedly supported the conclusion that maternal stress and elevated monoamines and glucocorticoids increase affective disorders and impair memory and stress-coping abilities in mammalian and avian offspring (Brown et al., 2000; Hayward and Wingfield, 2004; Markham et al., 2006; Talge et al., 2007; Kinsella and Monk, 2009). Early life stress or stress during maturation can result in higher reactivity to stressful events later in life, increased anxiety, and learning impairments (Fride et al., 1986; Weinstock, 1997; Lupien et al., 2009). These behavioral problems vary depending on the age of the subject at the time of exposure and the intensity of the causative stressor and may not appear in an individual until long after exposure to the stressor, sometimes manifesting after the individual has reached full maturity (Lupien et al., 2009). Alteration of affective states as well as the point at which behavioral changes and fear modification present during an individual's lifetime differ depending on type of fear and targets of elevated NE in the brain during development (Sahay and Hen, 2007). In our study, we accordingly found that poultry

exposed to elevated embryonic NE levels display altered behavioral expression at various points in later life.

Studies in chicks show that increased NE impacts arousal and cognitive and learning processes, encoding emotional memories that can contribute to survival and flight-or-fight responses (Murchison et al., 2004). Elevated NE can increase or decrease memory consolidation and discrimination abilities depending on the level of NE, the subject's developmental age, and the type and location of activated adrenergic receptors (Foote et al., 1983; Gibbs and Summers, 2002; Markham et al., 2006; Lupien et al., 2009). Our results are consistent with these findings. Quail showed dose- and age-dependent spatial memory ability, suggesting that elevated NE can influence long-term cognition as early as ED1. Varying maze performance between NE treatments at wk 4 and wk 8 may be attributed to differential development or activation of centrally-located adrenergic receptors. Norepinephrine's role in behavioral programming related to spatial memory and cognition carries implications for poultry management. Open floor housing systems, for instance, rely on the use of nest boxes for more efficient egg collection and fewer broken and dirty eggs (Appleby, 1984). However, birds must learn to use nest boxes and once a bird establishes a preferred location to lay eggs, it is difficult to change lay behavior (Pearl and Surface, 1909; Hurnik et al., 1973; Kite and Cumming, 1980). Our research shows that targeted manipulation of memory via elevated embryonic NE may improve memory abilities in ways that can contribute to learning applications in industry management. Further understanding of embryonic NE's specific roles in developing cognitive skills at critical points in development may improve memory-based training for easier management in intensive production systems.

Levels of peripheral NE impact the extent of behavioral expressions of fear. Low doses of 0.05 to 0.2 mg/kg NE injected into chicks have no effect on TI duration while high doses of 0.25, 0.5, or 1.0 mg/kg injected into chicks 15 minutes prior to testing increase TI duration. Tonic immobility duration increases with greater NE concentrations (Thompson et

al., 1974; Thompson and Joseph, 1978). While our data show that TI duration is impacted by NE administration at ED1, the time chicks remained in a feigned death stance tended to be greater in chicks that received lower doses of embryonic NE compared to higher doses, contrary to previous findings in experiments in which NE was administered post-hatch. Thompson and Joseph (1978) suggest that increased TI duration following NE injection is related to β-adrenergic receptor stimulation while Hennig et al. (1984) suggest that α_1-adrenergic receptor stimulation attenuates TI duration as a rebound effect following α_2-adrenergic receptor stimulation, which induces the immobile state. High and low doses of NE at the start of embryogenesis could differently impact development of the varying types of adrenergic receptors, leading to tendencies of higher TI duration in low dose chicks. Over time, treatment differences may be minimized due to compensatory fear-coping mechanisms in low dose birds including physiological changes such as in GABA-receptor density in response to constant human handling over time (Jones and Faure, 1981; Fluck et al., 1997). However, fear in neonatal chicks can lead to reduced growth, increased escape attempts and resulting injuries, and limited environmental adaptability and social interactions, severely impacting well-being and physical and behavioral development during vital stages of ontogenesis (Jones, 1996). Our findings thus highlight the need to understand how dose differences of *in ovo* NE deposition influence early life fear and handling responses.

Two to three day old chickens injected with 0.1 μg corticotropin-releasing factor (CRF) along with 12.5, 25.0, or 50.0 μg of NE show dose-dependent reduction in locomotion and distress vocalizations and increases in sitting with closed eyes in an open field isolation test conducted immediately following injection compared to chicks injected with 0.1 μg of CRF alone (Zhang et al., 2003).These dose-dependent differences in behavior are magnified over the span of minutes. Corticotropin-releasing factor interacts with NE in the LC and hypothalamus (HYP) to mediate behavioral responses to stress (Koob, 1999). In our research, when quail injected with high or low doses of NE at ED1 were placed in an open field

isolation test, high dose birds tended to transition between being stationary and ambulatory more frequently than other treatments and foraged significantly less than control birds. High and low dose birds tended to defecate more than control birds. These behavioral changes indicate a heightened state of anxiety. It is evident from these findings that elevated NE has both short- and long-term dose-dependent effects on fear-coping strategies in developing birds. Due to NE's regulatory interactions with other biological compounds associated with fear responses, such as CRF, elevated embryonic NE may produce these behavioral alterations directly or indirectly via an interacting neuropeptide.

Responses to social stress are regulated by the brain's LC and can influence proactive defense strategies (Cecchi et al., 2002; Bingham et al., 2011). Critical periods of development including the pre- and post-natal periods and early adolescence have profound effects on development of neuroendocrine systems related to stress-coping in social encounters (Plotsky et al., 2005; Romeo et al., 2006; Murgatroyd et al., 2009; Bingham et al., 2011). Twenty-four to seventy-two hrs following a 7-day resident-intruder induced social stress, rats in early adolescence exhibited age-dependent increases in proactive defensive burying and swim tests accompanied by elevated LC discharge rates that were inhibited by microinjection of a CRF antagonist (Bingham et al., 2011). Following immobilization stress, rats demonstrated anxious behavior with reduction in open-arm exploration in an elevated-plus maze and reduction of social behavior in a social interaction test (Cecchi et al., 2002). However, microinjections of the α_1-receptor antagonist, benoxathian, or a cocktail of β_1- and β_2-receptor antagonists, betaxolol and ICI 118,551, into the lateral bed nucleus of the stria terminalis were able to reverse the reduction of open-arm exploration but not the reduction of social interactions. These data provide support for α_2-receptor roles in social stress responses. In our study, low dose birds tended to attempt to escape more than control birds when placed with a novel conspecific showing avoidance of a social stress or greater variability in responses to

social stresses. Within the context of prior research, embryonic NE may alter forebrain α_2-receptor density and binding affinity leading to avoidance behavior.

In mice, meeting a novel conspecific or aggregating in one place leads to a decrease in brain NE and increased catabolic turnover (Bliss and Ailion, 1969). Rats that perform an aggressive behavior, such as biting, demonstrate attenuation of NE turnover and potential reward pathway activation (Tsuda et al., 1988; Haden and Scarpa, 2007). Results from our work provide support that NE plays a role in early embryonic development of the physiological systems involved in social stress responses that may be further altered at critical points in later life. We observed no differences in social, aggressive, or reproductive behaviors 24 and 36 hrs following a rehoming stress at early sexual maturity but less gentle feather pecking in NE-treated birds compared to control birds in later sexual maturity after several wks of exposure to a conspecific. Differences in stress-coping strategies in high dose and low dose NE-treated birds in a 20 minute novel conspecific test at 11 wks of age suggest that varying embryonic NE levels drive expression of proactive defensive versus aggressive responses to social stress. Adrenergic receptor and downstream neuroendocrine system development including feedback loops could impact NE metabolic turnover depending on *in ovo* NE concentrations. Our conclusions have practical implications for poultry management. Due to the high volume of poultry in commercial husbandry conditions, a bird's recognition of flock mates is often hindered. Lack of recognition of conspecifics interferes with important natural hierarchical behaviors and often leads to increased social stress and aggression (Guhl and Allee, 1944; Guhl and Ortman, 1953; Mauldin, 1992). Differences in pecking at and escaping from conspecifics in birds exposed to elevated levels of NE demonstrate that maternal stress can lead to variability in dominant and submissive interactions. Understanding embryonic NE's role in driving poultry reactions to social stress can help predict bird behavior and restructure management practices accordingly to reduce stress and prevent injuries resulting from aggression or escape attempts.

Our data from memory, TI, open field isolation, and conspecific tests collectively show that elevated embryonic NE impacts cognitive, fearful, aggressive, and social behaviors in a dose- and age-dependent manner with consequences for poultry management. Further knowledge of the precise physiological mechanisms driving these behavioral changes may provide insight into how embryonic NE can be manipulated to positively program behavior for improved animal well-being and better husbandry practices as well as how embryonic NE impacts the development of peripheral systems including the gut. Generally, an increase in NE metabolism during stress leads to increased NE metabolism and increased adrenergic activity (Camp, 2015). Increased adrenergic activity is offset by changes in α- and β- receptor density and binding affinity depending on type, duration, and predictability of the stressor as well as the demographics of the stressed subject (Tejani-Butt et al., 1994; Seo et al., 1999; Porterfield et al., 2012; Camp, 2015). Maternal stress can similarly lead to HPA-axis dysfunction and alteration of adrenergic receptor formation in offspring including decreased hippocampal synaptic density and increased Trp, 5-HT, and 5-HIAA (Peters, 1986; Weinstock, 1997; Hayashi et al., 1998; Hayward and Wingfield, 2004; Morilak et al., 2005; Talge et al., 2007; Lupien et al., 2009). The HIPP is involved in learning, anxiety behaviors, and memory encoding, especially relating to spatial memories (Bannerman et al., 2004). Norepinephrine promotes neurogenesis and activates neurogenic precursors via β_3 – adrenergic receptor binding in adults (Jhaveri et al., 2010). Based on the behavioral changes we observed in quail, it is possible that increased NE levels during embryogenesis carry consequences for proper adrenergic network formation. If pre-hatch development of adrenergic receptor networks is altered by elevated *in ovo* NE, it is likely that post-hatch development is also affected. The potential resulting variance in adrenergic network densities in diverse parts of the CNS and PNS throughout ontogenesis may lead to dose-dependent differences in cognitive, fear, aggression, and social behavioral responses over the course of maturation.

It has also been shown that the α-agonists clonidine and guanfacine diminish α-methyl-p-tyrosine-induced NE depletion (Zigun et al., 1981). Given that social and aggressive behaviors are associated with NE turnover, elevated embryonic NE's possible neurogenic effects on adrenergic receptor density could impact NE metabolism and feedback loops, leading to changes in behavior in post-hatch offspring (Bliss and Ailion, 1969; Tsuda et al., 1988; Haden and Scarpa, 2007), Changes in NE metabolism could further alter behavioral outputs induced by associated stress-response compounds including CRF, glucocorticoids, and other monoamines (Stene et al., 1980; Oades, 1985; Joels and De Kloet, 1989; Zhang et al., 2003). Our ongoing physiological research will provide evidence or the lack thereof of adrenergic network alteration including receptor density, anatomical sites of altered receptor development, NE metabolism turnover changes, and effects on downstream stress-response systems. Understandings of these physiological differences and how they translate into dose-and age-dependent influences on poultry cognition, fear, aggression, and social responses could clarify the role NE plays in behavioral programming in offspring, possibly allowing for targeted manipulation to improve bird well-being and husbandry management in the future.

High reactivity to stress and plasticity of physiological mechanisms responsible for producing stress responses may be rooted in increased biological sensitivity to certain contexts with detrimental or beneficial effects on long-term fitness depending on environment throughout ontogeny (Boyce and Ellis, 2005). The polyvagal theory of emotion further proposes that the autonomic nervous system evolves in response to affective and emotional experiences and social environment resulting in physiological and behavioral changes that lead to phylogenetic change and improve fitness and stress-coping abilities (Porges, 1997). Norepinephrine-treated quail displayed altered responses in fear, aggression, isolation, and social stress tests with high dose birds particularly impacted in multiple assessments. Increased *in ovo* deposition of NE due to maternal stress may increase offspring sensitivity to

stress-inducing conditions in order to improve long-term fitness conditional upon offspring being raised in supportive environments (Boyce and Ellis, 2005). Throughout the course of our study, however, birds were continually placed in stress-inducing situations that could have magnified biological alterations for coping with stress, preventing supportive environments to foster further development of stress-coping mechanisms. Future offspring studies may demonstrate that elevated embryonic NE can provide evolutionary benefits within subsequent generations given supportive environments in which to develop. Behavioral programming via elevated embryonic NE may thus prove instrumental in improving stress-coping abilities, affective states, and sociality in poultry breeding flock lines while considering both maternal and offspring environments.

Further research of NE's role in development of physiological networks mediating behavioral responses to stress will provide insight into how *in ovo* NE can be manipulated to improve health and coping abilities in poultry in the stressful environments of commercial production. Findings from our study provide clear evidence that elevated embryonic NE results in changes in behavioral expression pertaining to cognitive abilities, fear, aggression, and social responses in a dose- and age-dependent manner. Our data suggest that maternal stress has tangible impacts on coping abilities in offspring carrying implications for poultry well-being and management.

Chapter 3: The effects of embryonic norepinephrine on productivity, survival-related behaviors, and stress-coping mechanism in Japanese quail

Abstract

Our study evaluated the effects of elevated embryonic norepinephrine (NE) on productivity, behavior, and stress-coping mechanisms through weight, egg production, and scan sampling assessments. Results showed that increased *in ovo* NE impacts productivity, survival-related behaviors, and stress-coping mechanisms. Poultry breeding flocks experience high levels of stress from numerous sources including feed restrictions, confinement, social aggression, and changing environmental parameters. Maternal and incubation stress can have long-term impacts on physical and behavioral development in offspring via an increase in ovo catecholamine levels, including (NE). In order to determine the effects of elevated *in ovo* NE on productivity and survival-related behaviors, Japanese quail (*Coturnix japonica*) embryos were injected with 10 μL of 0.01M or 0.05M treatment concentrations of NE or saline at ED1 (n = 130) and incubated along with intact controls (n = 80). Average live body weights were taken at day 1, wk 6, and wk 11, prior to physiological sampling and organ weight data collection and also taken every other wk between wks 3 and 9. The daily number of eggs produced was recorded once the birds reached sexual maturity. Weekly behavioral scan samples were taken during the morning and evening from wk 4 (juvenile) to wk 11 (sexually mature) with birds rehomed between the wk 6 and wk 7 scan samples. Our results show no differences in average body weights at day 1, wk 6, and wk 11 ($P = 0.344$), testes ratios ($P = 0.659$) at wk 6, and organ to body weight ratios at wk 11 ($P = 0.5609$ heart, $P = 0.4112$ liver, $P = 0.454$ spleen). However, low dose birds experienced significantly lower body weights at wk 3 compared to saline birds ($P = 0.0007$). High and low dose birds exhibited reduced egg

production (P = 0.0008). Norepinephrine treatment had long-term impacts on survival-related behaviors and altered stress coping strategies in response to rehoming as evidenced by patterns of change in multiple behaviors including inactivity (P < 0.001), drinking (P = 0.036), foraging (P = 0.004), walking (P < 0.001), and other behaviors at wk 7. Scan samples showed significantly greater eating frequencies in mature high dose birds (P = 0.0217) and greater drinking frequencies in juvenile low dose birds compared to control birds (P = 0.0088). High dose birds foraged significantly less compared to low dose and saline birds (P = 0.0199). Norepinephrine-treated birds exhibited significantly reduced frequencies of walking compared to control birds (P = 0.0004) and significantly greater frequencies of inactivity compared to saline birds (P < 0.0001). Altered consumption and activity-related behaviors did not translate into greater weight gain or egg productivity, suggesting that increased *in ovo* NE at the start of embryonic development plays a role in programming of survival-related behaviors and stress-coping mechanisms in a dose- and age-dependent manner driven by changes in underlying adrenergic or metabolic mechanisms. Findings highlight a need for decreasing sources of maternal, incubation, and environmental stress in a commercial setting to improve poultry well-being.

Introduction

Avian behaviors are closely linked to physiological states and provide insight into a bird's health and well-being. Studying behaviors in agricultural birds can aid in discovering ways to promote well-being within the constraints of the poultry industry. Hormones and neurotransmitters such as norepinephrine (NE) act as mediators of behavior and physiology. Norepinephrine is a catecholamine implicated in arousal, fight or flight responses, and reactions to stress (Lichtenstein et al., 1984; Dienstbier, 1989; Morilak et al., 2005; Markham et al., 2006). The neurogenic catecholamine is also involved in normal embryonic and early life development although its precise role is not clear (Jhaveri et al., 2010). Mammalian and

avian research has suggested that maternal and incubation stresses can elevate NE and other monoamine and hormone levels in an embryo, resulting in short- and long-term affective behavioral and physiological alterations as well as impaired stress-coping abilities in developed offspring (Thompson, 1957; Fride and Weinstock, 1988; Alonso et al., 1991; Epple et al., 1997; Vallee et al., 1997; Secoli and Teixeira, 1998; Brown et al., 2000; Morley-Fletcher et al., 2003; Markham et al., 2006; Van den Bergh et al., 2008; Kinsella and Monk, 2009). Further study of embryonic NE's impact on a range of survival-related behaviors in offspring can highlight potential underlying mechanisms of NE's biological function and the overarching effects on industry productivity and bird health.

In order to examine the effects of elevated NE on poultry behavior, normal poultry ethology must first be understood. Wild and domestic avian behavior has been studied extensively with particular consideration given to applications for the poultry industry. Red Junglefowl are the evolutionary predecessors of domestic chickens. Poultry management should consider Red Junglefowl behaviors in understanding the ethology of domestic fowl and attempt to promote these natural behaviors in poultry to improve avian well-being. In a study by Dawekins (1989), Red Junglefowl were observed for 20 minutes every hour between the morning and evening roosting periods in a zoo setting. A time-budget analysis showed that although the birds were fed *ad libitum*, 60.6% of the time that the hens were in view, ground pecking behavior was observed and 34.1% of the time, ground scratching behavior was observed, both natural foraging-related behaviors. Walking, vigilance, preening, roosting, and standing were other common behaviors observed in the birds (Dawkins, 1989). Observations of Red Junglefowl in the wild and of feral fowl have concluded that much time is spent on foraging and reproductive activities similar to behaviors exhibited in domestic poultry (Johnson, 1963; Collias and Collias, 1967; Savory et al., 1978).

While feeding, foraging, reproductive, and other survival behaviors seen in wild and feral fowl are reflected in domestic poultry, a number of factors including strain of bird, age,

social aggression, confinement, aviary management, stocking density, inter-bird space, group size, and environmental enrichment can affect time budgeting (Nicol, 1989; Hansen, 1994; Keeling, 1994; Bubier, 1996; Aerni et al., 2000; Weeks and Nicol, 2006). In a study by Murphy and Preston (1988), broiler chickens aged 27-50 days were observed to spend 64% of an hr lying down punctuated by other behaviors ultimately resulting in 41 bouts of lying, indicating restlessness. Eating and drinking frequencies were variable and dustbathing or agonistic behaviors were not observed. In comparison, dustbathing, perching, nesting, and foraging are considered behavioral needs in laying hens (Weeks and Nicol, 2006). In turkeys, 6 pairs of males housed in pens starting at 3 wks of age were observed for twelve 24 hr periods between 4 and 22 wks of age with scan samples taken every 5 minutes (Sherwin and Kelland, 1998). Turkeys were largely inactive during periods of darkness. During periods of light, the time-budget allotment for drinking and pecking remained fairly constant while time-budget allotments for active behaviors such as feeding, sitting, standing, walking, strutting, and preening varied. As the birds aged, sleeping, environmental pecking, wing-flapping, and running became less frequent showing decreased interest in active and exploratory behaviors. Another time budget study of 140 turkey poults housed in pen groups of 10-11 birds and scan sampled between 1 day of age and 12 wks of age showed that sitting and sleeping remained constant, feeding declined then remained constantly low, standing and walking and drinking initially decreased then increased, and environmental pecking and preening initially increased then decreased demonstrating changes in active behaviors over time (Hughes and Grigor, 1996). A time budget study of Japanese quail placed in groups of 8-9 individuals per aviary filled with natural soil and vegetation found that quail spent substantial amounts of time on active behaviors and less time on survival-related and social behaviors although 48% of the time was spent under cover disproportional to the amount of area covered (Schmid and Wechsler, 1997). Overall, behaviors vary depending on environment and an individual bird's

strain and age, but across fowl species, much time is allotted to feeding, foraging, reproduction, and social and environmental pecking.

In addition to external factors that influence time-budgeting, internal physiological factors can contribute to behavioral frequencies and duration. Norepinephrine increases in response to stress can induce aggression and fear and alter feeding, activity levels, and reproduction at a systemic as well as behavioral level. Numerous studies have shown that the catcheloamine affects appetite in many species. In satiated rats, injection of NE into the paraventricular nucleus (PVN) increases prepandual drinking (1.0-4.2 ng of NE) and eating (5.6 and 16.9 ng of NE) regardless of light levels and cycles (Leibowitz, 1978; 1978). Chronic NE injections to the PVN stimulate feed intake in terms of meal quantity over meal frequency, resulting in an increase in body weight (Leibowitz et al., 1984). Alpha-adrenergic agonists used to treat affective disorders including clonidine, amitriptyline (AMIT), and chlorimipramine (CIMIP) also increase feed intake when injected into the PVN with rat subjects demonstrating a preference for carbohydrate-based diets over protein- and fat-based diets following injection (Leibowitz et al., 1985). However, NE injections to non-central parts of the hypothalamus (HYP) and regions outside of the HYP in rats have little to no effect on drinking and eating (Leibowitz, 1975; 1978).

Norepinephrine associated with responses to stress may also have an effect on reproductive behaviors. In mice, prenatally stressed female offspring have later vaginal openings, longer estrus cycles, and greater median quality receptivity scores than female offspring of unhandled control mice (Politch and Herrenkohl, 1984). Rat studies in comparison have shown no reproductive behavioral or morphological changes in prenatally stressed females (Ward, 1984). Male offspring of female rats that are nutritionally, environmentally, or chemically stressed during late gestation exhibit significantly decreased male sexual behavior and significantly increased female sexual behavior suggesting a demasculinization of reproductive traits (Ward, 1972; Masterpasqua et al., 1976; Rhees and

Fleming, 1981; Dörner et al., 1983; Ward, 1984). Male offspring of immobilized mothers exhibit an increased NE concentration in the preoptic area of the brain, contributing to a lack of sexual dimorphism that leads to long-term alterations in copulatory behavior (Reznikov and Nosenko, 1996). Supplementation of tyrosine methylester, a NE precursor, prior to immobilization stress in a late gestating rat counteracts the demasculating effects of prenatal stress on male offspring while decreasing social play in both males and females, suggesting that NE plays a vital role in development of sexual behaviors and characteristics (Ohkawa, 1987).

The known physiological effects of NE can alter patterns of key survival-related behaviors. However, little research has been conducted on the time-budgeting and productivity effects of elevated embryonic NE. Further investigation of the precise effect of elevated *in ovo* NE on a range of behaviors can provide insight into how to better meet industry goals of high productivity and efficient management while promoting poultry well-being. Injection of NE into quail eggs during early embryonic development may serve as a model for maternal and incubation stress in poultry. Subsequent observation of time-budgeting and physical differences can better highlight the effects of elevated *in ovo* catecholamines on feeding, reproductive, aggressive, social, and other survival-related behaviors that impact industry management and success.

Methods

Treatment

Fertilized Japanese quail (*Coturnix japonica*) eggs were collected for three wks and stored in the dark at 11°C prior to incubation. Based on prior literature values determined by Markham et al. (2006), 10 µl of 0.05M ("high dose", n = 130) or 0.01M ("low dose", n = 130) treatment concentrations of L- norepinephrine bitartrate salt monohydrate thoroughly

dissolved in sterile saline on the day of the injection or 10 µl of sterile saline (n = 130) were injected into the albumin of the fertilized eggs using ½ cc tuberculin syringes with 27 gauge needles. Treatment eggs were incubated along with intact controls (n = 80) to assess impacts of injection on hatchability. The procedure was performed over two replicates in time.

Husbandry

Injected eggs were incubated in a GQF 3258 Digital Control Sportsman® incubator and hatcher at 99.5°C and 70% humidity for 6-21 days. Extremely weak and deformed chicks were euthanized by cervical dislocation upon hatching while healthy, dry chicks were placed on a 24 hour light cycle in 95°F brooding cages within treatment groups (0.05M NE, 0.01M NE, saline, intact). Chicks were provided with water in inverted gravity water cups and Mazuri® Gamebird Starter feed on brown paper cage sheets ad libitum. Once the chicks were large enough, feed was placed in metal dishes. After all chicks in the replicate had hatched, the birds were sorted into smaller treatment groups of 5-6 individuals per cage.

The temperature in the cages was reduced by 5°F each wk until the cages were 75-80°F. At 4 wks of age, the established treatment groups of 5-6 birds were transferred from the brooding cages to breeding batteries at 70-75°F on a 12 hr light/dark cycle. The birds had ad libitum access to water from automatic drinkers and Mazuri® Gamebird Maintenance feed in troughs. Sex ratios were determined at the end of 6 wks of age and two males from each cage were sampled for physiological data collection. Females were reorganized into cages of 2-3 non-familiar conspecifics for further behavioral analysis. A final physiological sample collection for the females occurred at 11 wks of age.

Productivity

The number of eggs produced each day once sexual maturity was reached was recorded. At 3, 5, 7, and 9 wks of age all birds were individually weighed. At one day, 6 wks,

and 11 wks of age, 8 to12 birds were sampled at random for physiological data collection. Birds were first weighed then euthanized by cervical dislocation. At wk 6, the testes were weighed for a comparison of testes ratios. At wk 11, the heart, liver, and spleen were immediately removed to obtain organ weight to body weight ratios.

Behavioral Testing

Between wks 4 and 11, behavior was assessed using scan sampling techniques in the morning an hr after the lights were turned on and in the late afternoon four hrs before the lights were turned off on a 12 light/dark schedule. Eight scans were conducted per cage 5 minutes apart. Birds were observed for posture (sitting versus standing) and behavior (eating, drinking, foraging behavior, walking, inactive/resting behavior, vigilant behavior, preening, pecking at the cage, scratching the floor, gentle feather pecking, severe feather pecking, threats, and aggressive pecking; see Appendix I for ethogram).

Statistical Analysis

Results were analyzed for normality and transformed if needed using logarithmic or square root transformations. Egg productivity and scan sampling data were analyzed as repeated measures between wks 4 and 6 prior to rehoming and between wks 7 and 11 following rehoming. All data were analyzed for treatment, age, and replicate interactions and main effects using a mixed-model procedure for Analysis of Variance (ANOVA) and differences of least square means comparisons in SAS Studio software (SAS, Cary, NC). P-values for least square means comparisons were adjusted using a Sidak correction. P-values less than or equal to 0.05 were accepted as significantly different and P values greater than 0.05 and less than 0.100 were accepted as statistical trends.

Results

Hatch Rates

The strain of Japanese quail we used demonstrated a hatchability rate of approximately 30% in previous breeding operations. The overall hatchability rate for the birds in our study was 53%. In the first replicate, 176 eggs were incubated of which 51% of the high dose eggs, 65% of the low dose eggs, 53% of the saline eggs, and 42% of the intact eggs hatched. During the second replicate, 242 eggs were incubated of which 46% of the high dose eggs, 54% of the low dose eggs, 59% of the saline eggs, and 49% of the intact eggs hatched. Total hatchability rates were 48% for the high dose treatment, 60% for the low dose treatment, 56% for the saline treatment, and 45% for the intact treatment. We did not conduct enough replicates for statistical analysis of hatch rates. However, insertion of a needle into an egg did not appear to decrease hatchability rates as the intact treatment demonstrated lower hatch percentages in both replicates compared to the other treatments.

Weight

Average live body weights taken at times of physiological sampling at day 1, wk 6, and wk 11 increased as the birds developed (Table 6) with main effect differences in age ($P < 0.001$, $F_{2,108} = 3639.44$) but not in treatment ($P = 0.344$, $F_{2,108} = 1.12$). Within each treatment, birds weighed less at day one compared to wk 6 and wk 11 ($P < 0.001$) and less at wk 6 compared to wk 11 ($P < 0.01$ for each treatment). The left and right testes weights at wk 6 did not differ between treatments ($P = 0.5527$, $F_{3, 36} = 0.71$ and $P = 0.5874$, $F_{3, 36} = 0.65$, respectively). The ratio of left to right testes weights (Table 7) did not differ between treatments ($P = 0.659$, $F_{3, 35} = 0.54$). The heart weight to body weight, liver weight to body weight, and spleen weight to body weight ratios (Table 8) did not differ between treatments at

wk 11 ($P = 0.5609$, $F_{3, 40} = 0.69$; $P = 0.4112$, $F_{3, 40} = 0.98$; $P = 0.454$, $F_{3, 40} = 0.90$, respectively).

Table 6. Average body weight ± SEM at times of physiological sampling.

Treatment	Day 1		Wk 6		Wk 11	
	Average Body Weight±SEM	N	Average Body Weight±SEM	N	Average Body Weight±SEM	N
High	9.31 ± 0.59	8	190 ± 9	11	236 ± 13	14
Low	8.58 ± 0.45	8	178 ± 7	12	259 ± 13	12
Saline	10.23 ± 0.86	8	189 ± 7	12	269 ± 21	9
Intact	9.24 ± 0.63	8	182 ± 9	8	251 ± 18	12

Table 7. Average testes weights ±SEM and testes ratios ±SEM at wk 6.

Treatment	Average L Testes Weight ±SEM	Average R Testes Weight±SEM	Testes Ratio ±SEM	N
High	0.655 ± 0.300	0.779 ± 0.379	0.940 ± 0.041	10
Low	1.225 ± 0.337	1.356 ± 0.373	0.969 ± 0.079	11
Saline	1.275 ± 0.426	1.241 ± 0.399	0.976 ± 0.050	12
Intact	0.691 ± 0.408	0.753± 0.468	1.038± 0.067	7

Table 8. Average organ weight to average body weight ratios ±SEM at wk 11.

Treatment	Heart:Body Weight±SEM	N	Liver:Body Weight±SEM	N	Spleen:Body Weight±SEM	N
High	0.0093 ± 0.0032	12	0.0305 ± 0.0100	12	0.0006 ± 0.0004	10
Low	0.0080 ± 0.0009	12	0.0274 ± 0.0088	12	0.0009 ± 0.0004	7
Saline	0.0082 ± 0.0019	9	0.0276 ± 0.0058	9	0.0005 ± 0.0002	5
Intact	0.0085 ±.0014	11	0.0317 ± 0.0078	11	0.0008 ± 0.0005	8

Average body weights taken every two wks between 3 and 9 wks of age increased through sexual maturity (Table 9) with main effect differences between treatments ($P = 0.0007$, $F_{3,268} = 5.86$) and ages ($P < 0.001$, $F_{3,268} = 155.72$). Saline birds tended to weigh more than high dose ($P = 0.069$) and intact ($P = 0.083$) birds and weighed significantly more than low dose birds ($P = 0.005$) with treatment-age interactions between saline and low dose birds at wk 3 ($P = 0.021$). Smaller body weights were observed in juvenile versus mature birds in every treatment, as expected. There were differences within each treatment at wk 3 compared to wks 5, 7, and 9 ($P < 0.001$), in saline birds at wk 5 compared to wk 7 ($P = 0.034$), and in low dose, saline, and intact birds at wk 5 compared to wk 9 ($P = 0.006$, $P = 0.006$, $P = 0.029$, respectively).

Table 9. Average body weights ± SEM throughout development.

Treatment	Wk 3		Wk 5		Wk 7		Wk 9	
	Average Body Weight ± SEM	N	Average Body Weight ± SEM	N	Average Body Weight ± SEM	N	Average Body Weight ± SEM	N
High	132 ± 3	24	192 ± 6	22	197 ± 13	8	222 ± 13	6
Low	127 ± 3	30	181 ± 5	30	198 ± 9	13	222 ± 12	13
Saline	142 ± 3	33	196 ± 5	31	225 ± 9	14	238 ± 11	14
Intact	132 ± 4	17	182 ± 7	15	208 ± 17	7	223 ± 17	7

Egg Productivity

The number of birds contributing to egg data collection varied throughout the study (Table 10). High and low dose NE-treated birds exhibited a delay in egg productivity as they reached sexual maturity (Figure 20) with differences in treatment-age interactions ($P = 0.0008$, $F_{15, 1088} = 2.58$). Norepinephrine-treated birds exhibited reduced egg production in both replicates compared to saline control and intact birds, but all birds exhibited delayed egg production in the second replicate compared to the first replicate. At wk 6, high dose birds produced the fewest eggs (0.04 ± 0.10) followed by intact birds (0.06 ± 0.11), low dose birds (0.15 ± 0.08), and saline birds (0.35 ± 0.08) with differences between the high dose and saline treatments ($P = 0.025$). At wk 7, intact birds produced the fewest eggs (0.07 ± 0.07) followed by high dose birds (0.09 ± 0.06), low dose birds (0.23 ± 0.05), and saline birds (0.38 ± 0.05). There was a tendency for high dose birds to produce fewer eggs compared to low dose birds ($P = 0.0733$). High dose, low dose, and intact birds produced fewer eggs compared to saline birds ($P < 0.0001$, $P = 0.0048$, $P < 0.0001$, respectively) and intact birds produced fewer eggs compared to low dose birds ($P = 0.0036$). At wk 8, high dose birds produced the fewest eggs (0.19 ± 0.06) followed by intact birds (0.27 ± 0.07), low dose birds (0.29 ± 0.05), and saline birds (0.43 ± 0.05). There were differences between the high and low treatment ($P = 0.0072$) and between the saline treatment and high, low, and intact treatments ($P < 0.0001$, $P = 0.0143$, $P = 0.0290$). At wk 9, the treatments produced approximately the same number of eggs (0.36 ± 0.07 high dose, 0.35 ± 0.06 low dose, 0.37 ± 0.06 saline, 0.35 ± 0.07 intact) with

no treatment differences. At wk 10, high dose birds produced fewer eggs (0.32 ± 0.06) than saline birds (0.47 ± 0.05; P = 0.0185). Low dose birds (0.36 ± 0.06) produced slightly more eggs than high dose birds and intact birds produced slightly more eggs than low dose birds (0.40 ± 0.07). At wk 11, high dose birds produced the fewest eggs (0.29 ± 0.08) followed by intact birds (0.36 ± 0.09), low dose birds (0.39 ± 0.07), and saline birds (0.44 ± 0.07) with no differences between treatments.

Table 10. N-values of birds contributing to lay data collection by the end of each wk. N refers to individual birds.

Treatment	Wk 6	Wk 7	Wk 8	Wk 9	Wk 10	Wk 11
High	39	15	15	15	15	15
Low	48	24	23	23	23	23
Saline	48	21	21	21	21	21
Intact	12	12	12	12	12	12

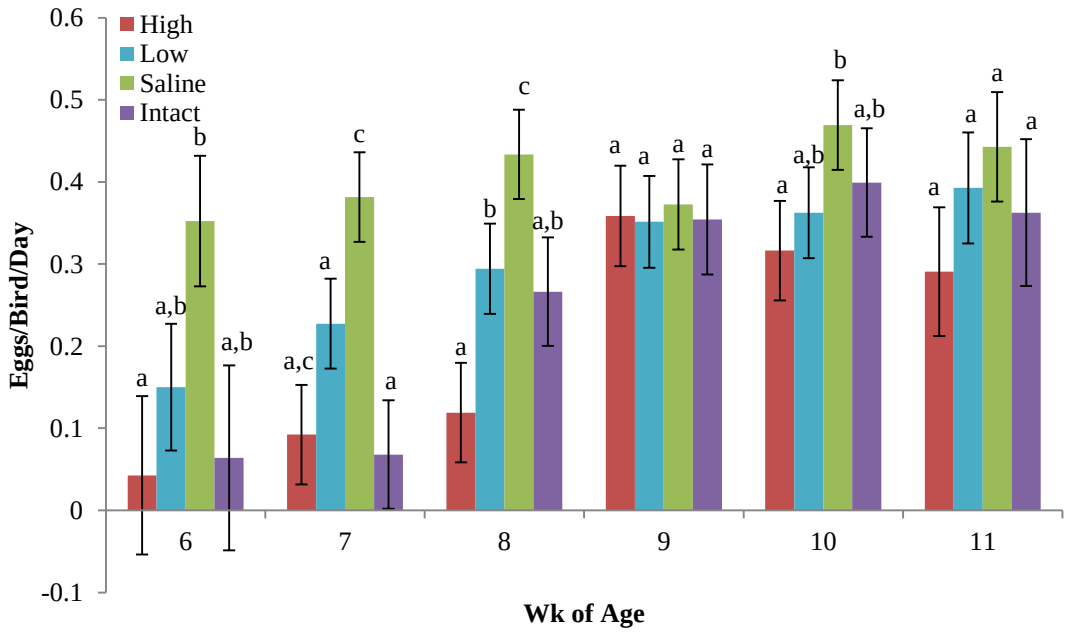

Figure 20. Number of eggs produced per bird per day. Different letters denote a significant difference between treatments (P < 0.05).

Scan Samples

The number of cage units scan sampled remained consistent from wks 4 to 6 and from wks 7 to 11 following rehoming (Table 11). Frequencies were calculated as percentages of birds performing an activity per cage to account for slight changes in the number of birds

housed due to mortality or removal from the study. Data were analyzed as repeated measures within wks 4 to 6 and within wks 7 to 11 for AM and PM scan samples.

Table 11. N-values of birds contributing scan sampling data. N-values refer to cage units.

Treatment	Wk 4	Wk 5	Wk 6	Wk 7	Wk 8	Wk 9	Wk 10	Wk 11
High	7	7	7	7	7	7	7	7
Low	9	9	9	12	11	11	11	11
Saline	9	9	9	11	11	11	11	11
Intact	5	5	5	5	5	5	5	5

High dose and low dose birds generally demonstrated higher frequencies of eating compared to control birds with a decrease in eating frequency among low dose birds at wk 7 following rehoming and an increases frequency of eating in mature NE birds at the AM scan samples (Figure 21). In the AM scan samples, there were replicate-treatment-age interaction trends ($P = 0.0629$, $F_{11, 172} = 1.77$) for wk 4 to wk 6 and differences in treatment-age interactions ($P = 0.0418$, $F_{12, 1347} = 1.81$) for wk 7 to wk 11. At the wk 10 AM scan sample, high dose birds ate significantly more frequently than saline birds ($P = 0.005$) and tended to eat more frequently than intact birds. At the wk 11 AM scan sample, high dose birds ate more frequently than intact birds ($P = 0.0120$) and low dose birds tended to eat less than intact birds ($P = 0.0850$). Low dose birds tended to eat less frequently at wk 4 than wk 5 ($P = 0.0924$) and ate less frequently at wk 9 than wk 10 ($P = 0.0198$). High dose birds tended to

eat less frequently at wk 8 than wk 10 (P = 0.0984).

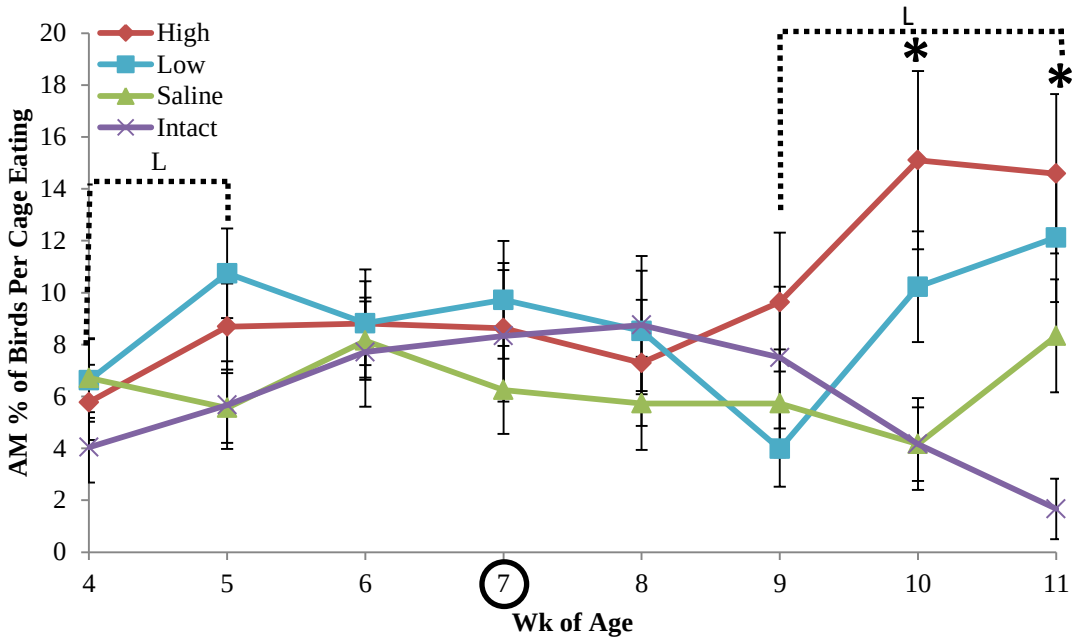

Figure 21. Percentage of birds per cage eating during the AM scan samples. * denotes significance between treatments within wks (P < 0.05). Dashed lines indicate differences within treatments between wks with the first letter of the respective treatment noted above (P < 0.05).

High dose birds demonstrated a higher average of birds per cage eating in the PM scan samples with a gradual increase over time (Figure 22, see Appendix II for values). In the PM scan samples, there were differences in treatment-age interactions (P = 0.0217, $F_{6, 676}$ = 2.49) for wk 4 to wk 6 and main effects of treatment (P = 0.0032, $F_{3, 72.8}$= 5.02) for wk 7 to wk 11. At the wk 5 PM scan sample, high dose birds ate more frequently than intact (P = 0.0255) birds. At the wk 10 PM scan sample, high dose birds ate more frequently than low dose birds (P = 0.0326) and tended to eat more frequently than intact birds (P = 0.0614). High dose birds ate less frequently at wk 4 than wk 5 (P = 0.0006) and wk 6 (P = 0.0021).

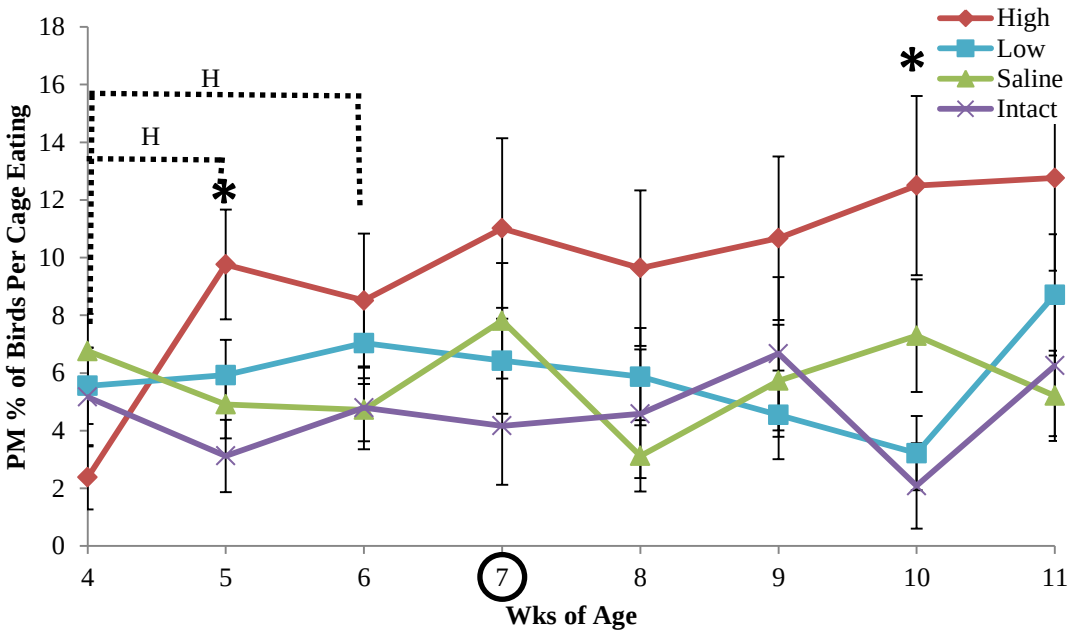

Figure 22. Percentage of birds per cage eating during the PM scan samples. * denotes significance between treatments within wks ($P < 0.05$). Dashed lines indicate differences within treatments between wks with the first letter of the respective treatment noted above ($P < 0.05$).

Low dose birds appeared to drink more frequently as juveniles but decreased time allotted to drinking in maturity while saline and intact birds demonstrated an increase in drinking frequency at wk 7 for the AM scan samples (Figure 23, see Appendix II for values). However, there were no differences in main effects or treatment-age interactions for wk 4 to wk 6 ($P = 0.9785$, $F_{6, 674} = 0.19$) or for wk7 to wk 11 ($P = 0.644$, $F_{12, 1351} = 0.81$).

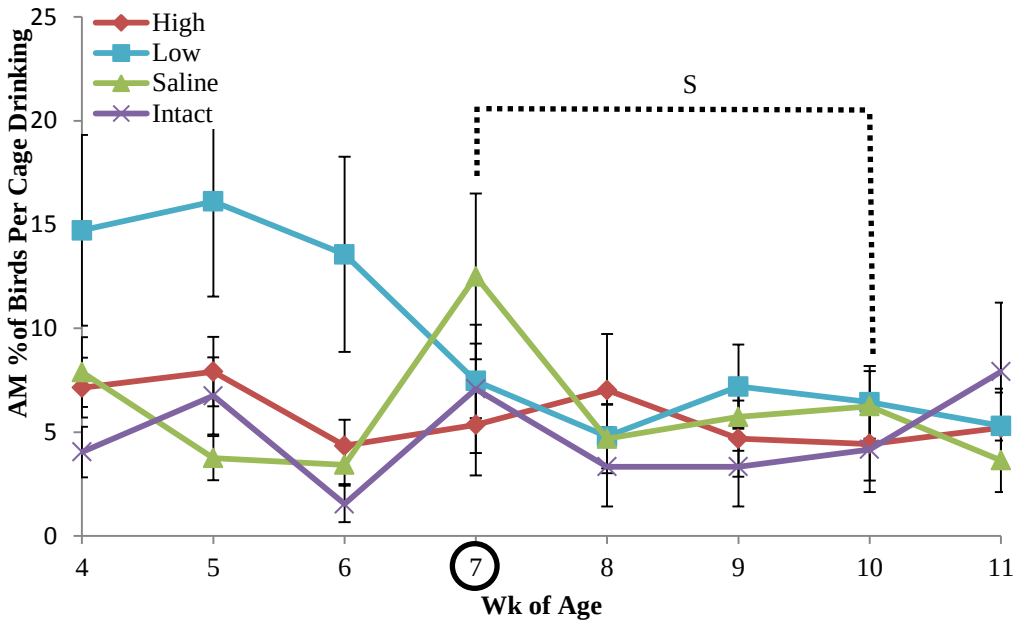

Figure 23. Percentage of birds per cage drinking during the AM scan samples. There were no differences between treatments within wks ($P < 0.05$). Dashed lines indicate differences within treatments between wks with the first letter of the respective treatment noted above ($P < 0.05$).

Low dose appeared to drink more frequently as juveniles before decreasing in time allotted to drinking at wk 6 then increasing at wk 7 for the PM scan samples. Other treatments remained fairly consistent in drinking frequency throughout growth (Figure 24, see Appendix II for values). In the PM scan samples, there were main effects of treatment ($P = 0.0088$, $F_{3, 152} = 4.01$) for wk 4 to wk 6 and trends in age effects ($P = 0.0644$, $F_{4, 1194} = 2.22$) for wk 7 to wk 11. At the wk 4 PM scan sample, low dose birds drank more frequently than high dose birds ($P = 0.0137$). At the wk 5 PM scan sample, low dose birds drank more frequently than high dose birds ($P = 0.0060$). Low dose birds drank more at wk 7 than wk 8 ($P = 0.0198$).

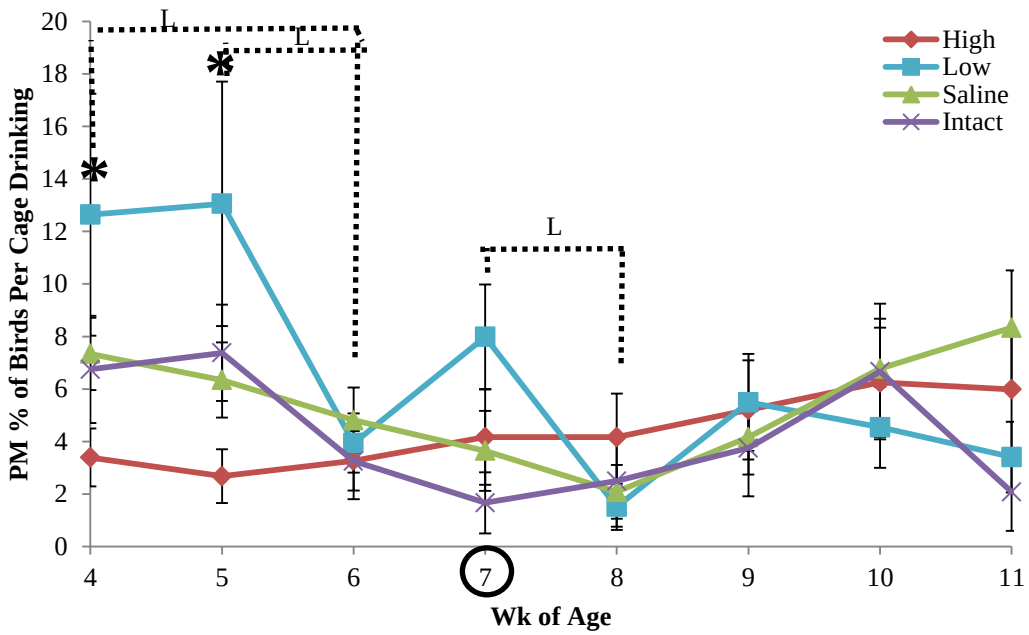

Figure 24. Percentage of birds per cage drinking during the PM scan samples. * denotes significance between treatments within wks ($P < 0.05$). Dashed lines indicate differences within treatments between wks with the first letter of the respective treatment noted above ($P < 0.05$).

High dose birds foraged consistently less frequently than the other treatments throughout the AM scan samples with slight increases in the percentage of birds foraging among all treatments over time (Figure 25, see Appendix II for values). In the AM scan samples, there were trends in treatment-age interactions ($P = 0.0999$, $F_{6, 682} = 1.78$) for wk 4 to wk 6 and differences in treatment-age interactions ($P = 0.0199$, $F_{12, 1354} = 2.02$) for wk 7 to wk 11. At the wk 6 AM scan sample, high dose birds foraged less than saline birds ($P = 0.0436$). At the wk 8 scan sample, low dose birds foraged more frequently than high dose birds ($P = 0.0078$). At the wk 10 AM scan sample, intact birds foraged more frequently high dose birds ($P = 0.0290$) and low dose birds ($P = 0.0113$). Saline birds tended to forage less frequently at wk 4 than wk 5 ($P = 0.0697$) and demonstrated significantly less foraging at wk 4 than wk 6 ($P = 0.0229$). Low dose birds foraged significantly less at wk 7 than wk 8 ($P = 0.0479$) and wk 11 ($P = 0.0286$). Intact birds foraged less at wk 7 than wk 10 ($P = 0.0040$) and tended to forage more at wk 10 than wk 8 ($P = 0.0641$) and wk 9 ($P = 0.0762$).

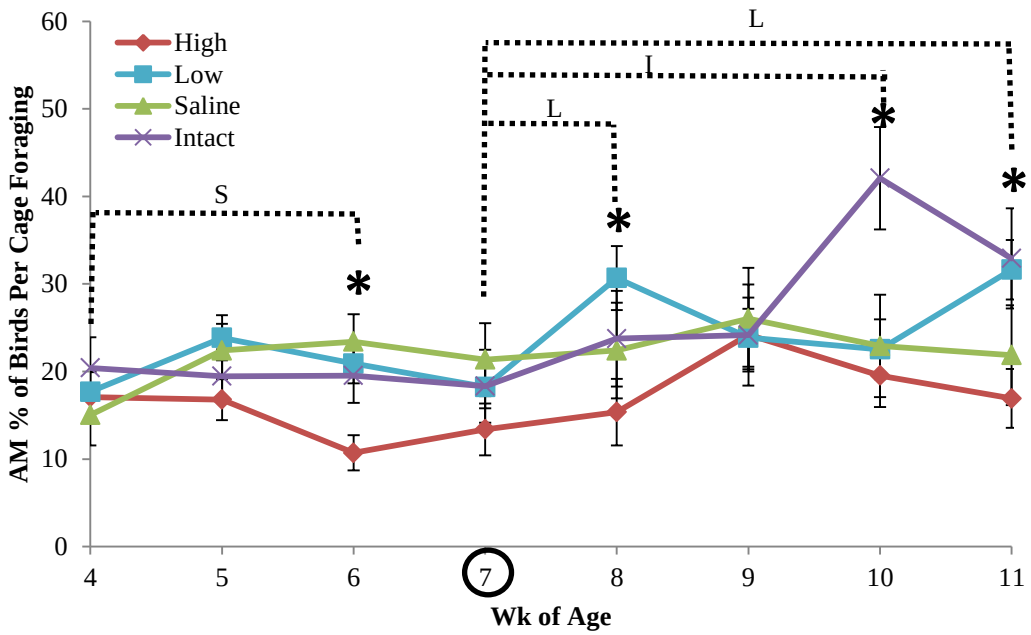

Figure 25. Percentage of birds per cage foraging during the AM scan samples. * denotes significance between treatments within wks ($P < 0.05$). Dashed lines indicate differences within treatments between wks with the first letter of the respective treatment noted above ($P < 0.05$).

High dose birds appeared to forage less frequently compared to other treatments in the PM scan samples (Figure 26, see Appendix II for values). High dose and intact birds exhibited a decrease while low dose birds exhibited an increase in average percentage of birds foraging at the wk 7 scan sample following rehoming. In the PM scan samples, there were main effects of age ($P < 0.001$, $F_{2, 688} = 11.92$) for wk 4 to wk 6 and main effects of treatment ($P < 0.001$, $F_{3, 469} = 2.02$) for wk 7 to wk 11. At the wk 7 PM scan sample, low dose birds foraged more frequently than high dose birds ($P = 0.0060$). At the wk 8 PM scan sample, low dose birds foraged less frequently than high dose birds and tended to forage less frequently than saline birds ($P = 0.0534$). Saline birds foraged less at wk 4 than wk 6 ($P = 0.0323$) and intact birds foraged less at wk 4 than at wk 5 ($P = 0.0270$) and wk 6 ($P = 0.0060$).

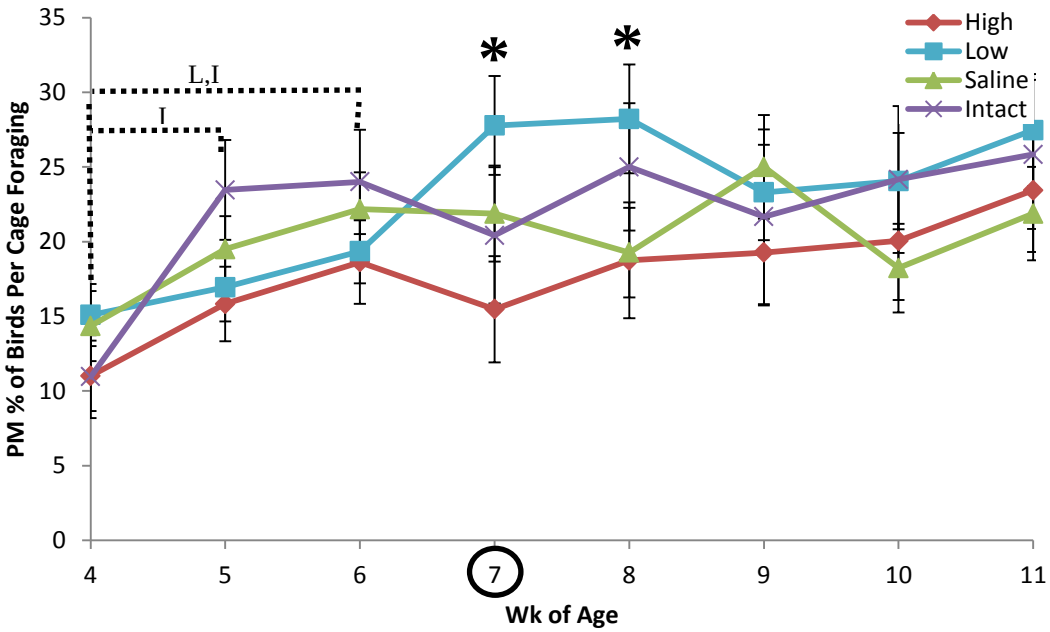

Figure 26. Percentage of birds per cage foraging during the PM scan samples. * denotes significance between treatments within wks ($P < 0.05$). Dashed lines indicate differences within treatments between wks with the first letter of the respective treatment noted above ($P < 0.05$).

Walking during the AM scan samplings increased as the birds matured with the control birds overall demonstrating a greater average percentage of birds per cage walking compared to NE-treated birds (Figure 27, see Appendix II for values). In the AM scan samples, there were differences in treatment-age interactions for wk 4 to wk 6 ($P = 0.0006$, $F_{6, 682} = 4.03$) and for wk 7 to wk 11 ($P = 0.0004$, $F_{12, 1362} = 2.99$). At the wk 5 AM scan sample, saline birds walked more frequently than high dose ($P = 0.0267$), low dose ($P = 0.0048$) and intact ($P = 0.0261$) birds. Intact birds walked less frequently at wk 4 than wk 6 ($P = 0.0481$). Low dose birds walked less frequently at wk 4 than wk 6 ($P = 0.0161$) and more frequently at wk 7 than wks 8 ($P = 0.0020$), 10 ($P = 0.0040$), and 11 ($P <0.0001$). Low dose birds had a tendency to walk less frequently at wk 7 than wk 9 ($P = 0.0706$). High dose birds tended to walk less frequently at wk 9 than wk 8 ($P = 0.0631$) and walked less frequently at wk 9 compared than wk 11 ($P = 0.0149$). Saline birds walked more frequently at wk 5 than wk 4 ($P< 0.0001$) and wk 6 ($P = 0.0006$).

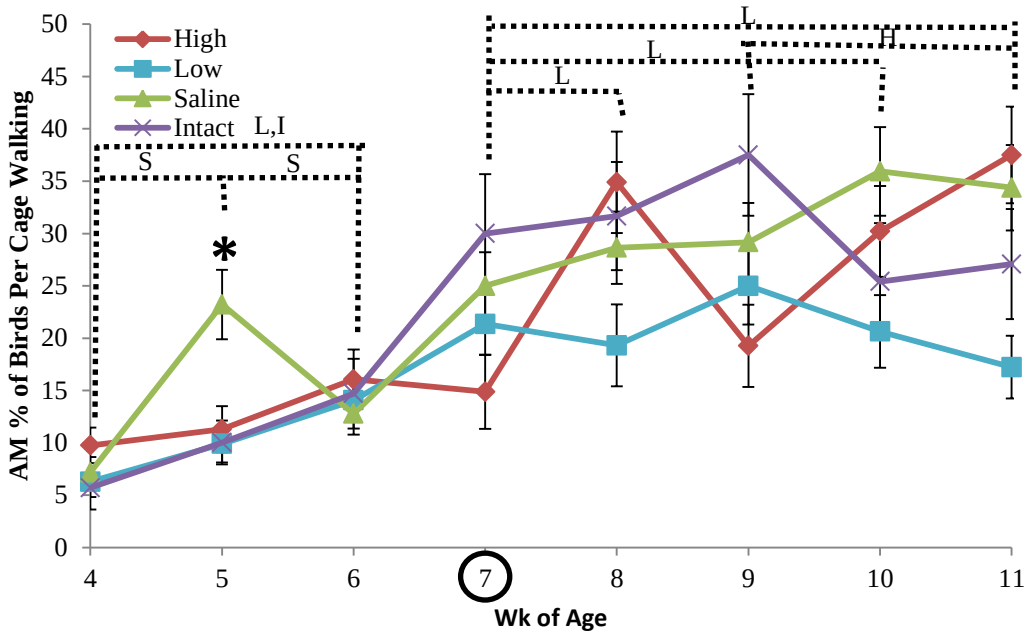

Figure 27. Percentage of birds per cage walking during the AM scan samples. * denotes significance between treatments within wks ($P < 0.05$). Dashed lines indicate differences within treatments between wks with the first letter of the respective treatment noted above ($P < 0.05$).

Walking during the PM scan samplings increased gradually as the birds matured with the control birds generally demonstrating a greater average of birds per cage walking compared to low dose birds (Figure 28, see Appendix II for values). In the PM scan samples, there were differences in treatment-age interactions ($P = 0.0237$, $F_{6, 687} = 2.45$) for wk 4 to wk 6 and main effects of wk ($P < 0.0001$, $F_{4, 1205} = 6.11$) for wk 7 to wk 11. At the wk 5 AM scan sample, high dose birds walked less frequently than saline birds ($P = 0.0413$). Low dose birds walked more frequently at wk 7 than wk 4 ($P = 0.0015$) and wk 5 ($P= 0.0472$). High dose birds tended to walk less frequently at wk 5 than wk 6 ($P = 0.0594$). Saline birds tended to walk less frequently at wk 7 than wk 10 ($P = 0.0678$) and wk 11($P = 0.0678$) and walked less frequently at wk 8 than wk 10 ($P = 0.0228$) and wk 11 ($P = 0.0228$).

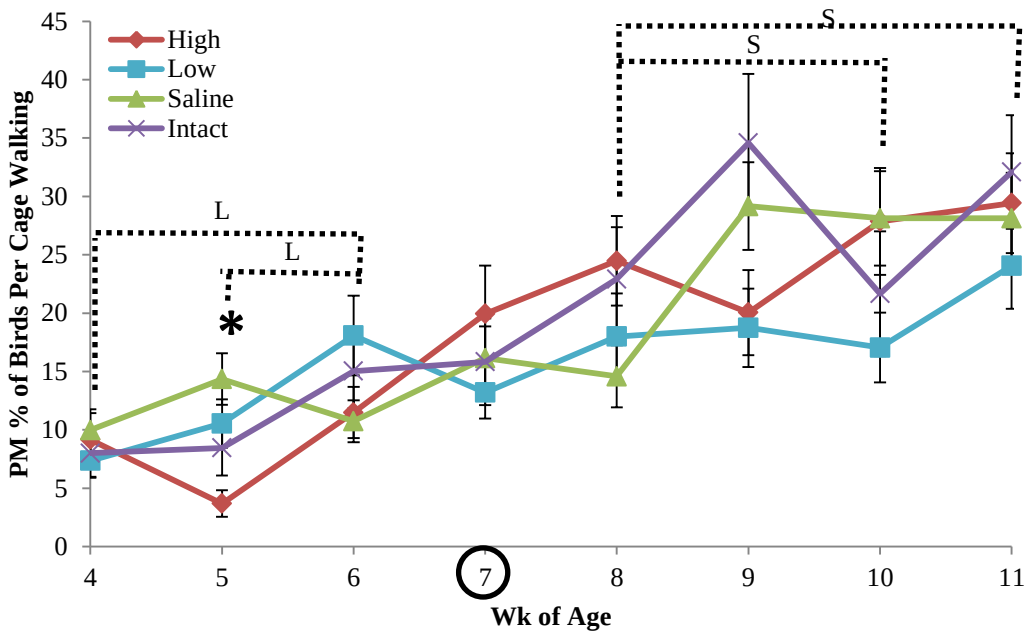

Figure 28. Percentage of birds per cage walking during the PM scan samples. * denotes significance between treatments within wks ($P < 0.05$). Dashed lines indicate differences within treatments between wks with the first letter of the respective treatment noted above ($P < 0.05$).

The average percentage of birds inactive decreased gradually over time for every treatment overall during the AM scan samplings, but the high dose and low dose birds increased sharply at wk 7 (Figure 29, see Appendix II for values). In the AM scan samples, there were main effects of age ($P < 0.0001$, $F_{2, 682}=39.28$) for wk 4 to wk 6 and differences in treatment-age interactions ($P < 0.0001$, $F_{12, 1360} = 4.57$) for wk 7 to wk 11. At the wk 7 AM scan sample, high dose birds were more inactive than saline ($P = 0.0018$) and intact ($P = 0.0048$) birds. Low dose birds were more inactive than saline birds ($P = 0.0267$) and tended to be more inactive than intact birds ($P = 0.0687$). High dose birds were significantly more inactive at wk 4 than wk 5 ($P = 0.0045$) and wk 6 ($P = 0.0140$) and at wk 7 than at wks 8 ($P = 0.0306$), 10 ($P < 0.0001$), and 11 ($P < 0.0001$). High birds tended to be more inactive at wk 7 than wk 9 ($P = 0.00650$). Low dose birds were more inactive at wk 4 than wks 5 and 6 ($P < 0.0001$ for both wks) and at wk 7 than wks 8 ($P < 0.0001$), 9 ($P = 0.001$), 10 ($P = 0.0060$),

and 11 (P = 0.0451). Saline birds were more inactive at wk 4 than wks 5 and 6 (P < 0.0001) and at wk 9 than wk 7 (P = 0.0188).

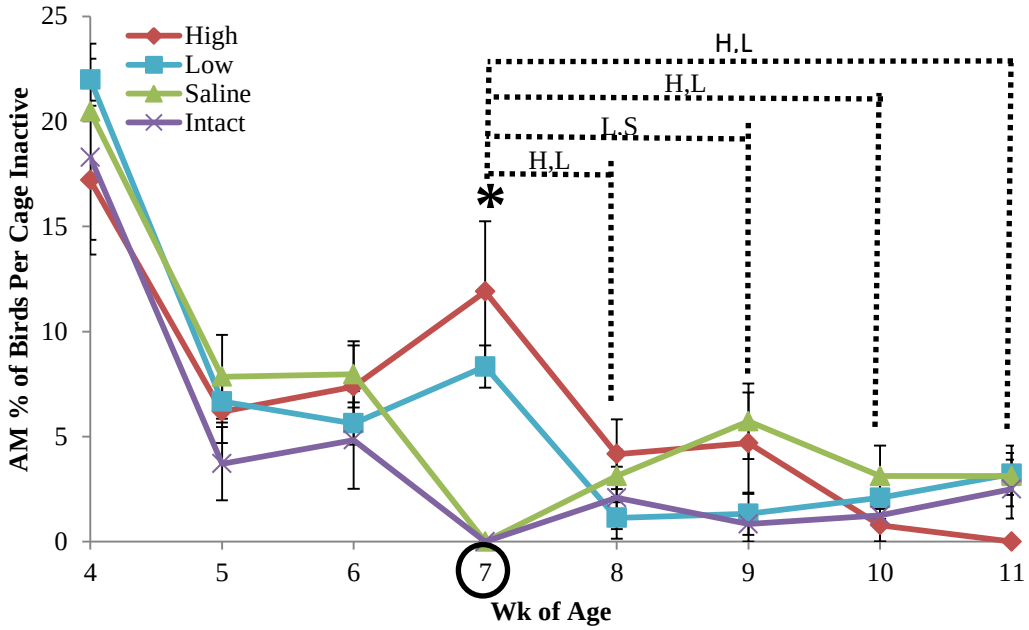

Figure 29. Percentage of birds per cage inactive during the AM scan samples. * denotes significance between treatments within wks (P < 0.05). Dashed lines indicate differences within treatments between wks with the first letter of the respective treatment noted above (P < 0.05).

During the PM scan samplings, the average percentage of birds per cage inactive gradually decreased as the birds matured (Figure 31, see Appendix II for values). High dose birds initially demonstrated a greater average percentage of birds inactive. The average percentages of saline and intact birds inactive increased during wk 7 while high dose birds increased at wk 8. In the PM scan samples, there were differences in treatment-age interactions for wk 4 to wk 6 (P = 0.0267, $F_{6, 684} = 2.11$) and for wk 7 to wk 11 (P < 0.0001, $F_{12, 1151} = 3.61$). At the wk 4 PM scan sample, high dose birds were more inactive than low dose birds (P = 0.0267). At the wk 7 PM scan sample, intact birds were more inactive than high dose (P = 0.0072) and low dose (P = 0.0024) birds. At the wk 8 PM scan sample, high dose birds were more inactive than low dose (P = 0.0024), saline (P = 0.0066), and intact (P = 0.0042) birds. At the wk 9 PM scan sample, high dose birds tended to be more inactive than

low dose birds (P = 0.0755). High dose birds were more inactive at wk 4 than wk 5 (P = 0.0075) and wk 6 (P < 0.0001) and at wk 5 than wk 6 (P = 0.0015). Low dose birds were less inactive at wk 6 than wk 4 (P = 0.0003) and wk 5 (P = 0.0134). Saline birds were more inactive at wk 4 than wk 5 (P = 0.0429) and wk 6 (P = 0.0006). Intact birds were more inactive at wk 4 than wk 5 (P = 0.0282) and tended to be more inactive at wk 4 than wk 6 (P = 0.0516).

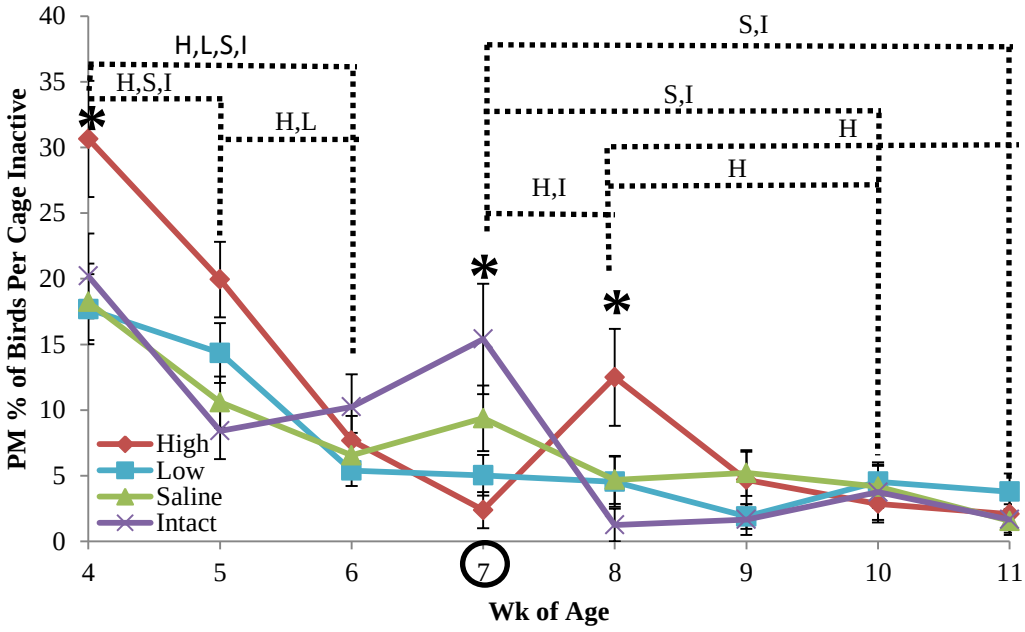

Figure 30. Percentage of birds per cage inactive during the PM scan samples. * denotes significance between treatments within wks (P < 0.05). Dashed lines indicate differences within treatments between wks with the first letter of the respective treatment noted above (P < 0.05).

The quail demonstrated a preference for standing rather than sitting during both the AM and the PM scan samples throughout the study. At both the AM and PM scan samplings, a high average percentage of birds from each treatment were standing with increases after wk 4 (Table 12). In the AM scan samples, there were main effects of age (P < 0.0001, $F_{2, 681}$ = 40.27) for wk 4 to wk 6 and differences in treatment-age interactions (P =0.0002, $F_{12, 1364}$ = 3.15) for wk 7 to wk 11. Intact birds tended to stand more than high dose (P = 0.0822) and low dose (P = 0.0828) birds at wk 7. High dose birds stood less at wk 4 than wk 5 (P =

0.0018) and at wk 7 than wk 8 (P = 0.0460), wk 9 (P < 0.0001), wk 10 (P = 0.0010), and wk 11 (P < 0.0001). Low dose birds stood less at wk 4 than wk 5 (P < 0.0001) and wk 6 (P < 0.0001) and at wk 7 than wk 8 (P = 0.0070), 9 (P < 0.0001), and 11 (P < 0.0001). Low dose birds stood more at wk 7 than wk 10 (P = 0.0010). Saline birds stood less at wk 4 than wk 5 (P < 0.0001) and wk 6 (P < 0.0426). Intact birds stood less at wk 4 than wk 5 (P < 0.0001) and wk 6 (P < 0.0006).

Table 12. Average percentage ± SEM of birds standing at the AM scan samples.

		High	Low	Saline	Intact
Wk 4	**% ± SEM**	74.4 ± 4.1	69.0 ± 3.5	73.6 ± 3.6	71.1 ± 5.0
	N	7	9	9	5
Wk 5	**% ± SEM**	89.8 ± 2.3	72.0 ± 3.1	90.0 ± 2.4	91.9 ± 2.3
	N	7	9	9	5
Wk 6	**% ± SEM**	84.1 ± 3.3	81.3 ± 2.7	83.2 ± 2.6	91.3 ± 2.6
	N	7	9	9	5
Wk 7	**% ± SEM**	84.5 ± 4.2	80.9 ± 2.9	94. 3 ±1.6	98.3 ± 1.2
	N	7	12	12	5
Wk 8	**% ± SEM**	94.5 ± 2.3	89.3 ± 2.3	93.8 ± 1. 9	98.3 ± 1.2
	N	7	11	12	5
Wk 9	**% ± SEM**	94.5 ± 2.5	88.6 ± 2.8	91.7 ± 2.1	98.8 ± 0.9
	N	7	11	12	5
Wk 10	**% ± SEM**	97.9 ± 1.2	78.6 ± 3.6	94.3± 1.8	98.8 ± 1.3
	N	7	11	12	5
Wk 11	**% ± SEM**	100 ± 0.0	85.8 ± 2.8	96.4 ± 1.3	95.4 ± 2.0
	N	7	11	12	5

At the PM scan samples, the percentage of birds standing increased over time with slight decreases among intact birds at wk 7 and saline birds at wk 10 (Table 13). There were main effects of age (P < 0.0001, $F_{2, 686}$ = 19.95) for wk 4 to wk 6 and trends in treatment-age interactions (P = 0.0686, $F_{12, 1215}$ = 1.67) and main effects of treatment (P = 0.0018, $F_{3, 522}$ = 5.09) for wk 7 to wk 11. High dose birds tended to stand less than intact birds at wk 8 (P = 0.0850). Low dose birds tended to stand less than saline birds (P = 0.0659) and stood significantly less than intact birds (P = 0.0040). Low dose birds tended to stand less at wk 4 than wk 5 (P = 0.0711) and wk 6 (P = 0.0588) and stood significantly less at wk 8 than wk 10 (P = 0.0267). High dose birds stood significantly less at wk 4 than wk 6 (P < 0.0001). Intact birds stood significantly less at wk 4 than wk 5 (P = 0.0108) and wk 6 (P = 0.0006) and at wk

7 than wks 8 (P <0.0001), 10, (P = 0.0040), and 11 (P = 0.0129). Intact birds tended to stand less at wk 7 than wk 9 (P = 0.0864).

Table 13. Average percentage ± SEM of birds standing at the PM scan samples,

		High	Low	Saline	Intact
Wk 4	**% ± SEM**	53.7 ± 4.6	69.4 ± 3.9	68.4 ± 4.2	54.4 ± 4.6
	N	7	9	9	5
Wk 5	**% ± SEM**	66.5 ± 3.5	72.0 ± 3.1	75.3 ± 2.9	73.2 ± 4.2
	N	7	9	9	5
Wk 6	**% ± SEM**	77.4 ± 3.2	81.3 ± 2.7	77.4 ± 3.0	77.1 ± 3.9
	N	7	9	9	5
Wk 7	**% ± SEM**	82.7 ± 3.8	80.9 ± 2.9	87.0 ± 2.9	74.2 ± 4.5
	N	7	12	12	5
Wk 8	**% ± SEM**	90.6 ± 3.1	89.4 ± 2.3	91.7 ± 2.1	96.7 ± 1.6
	N	7	11	12	5
Wk 9	**% ± SEM**	92.2 ± 2.5	88.6 ± 2.8	92.2 ± 2.0	97.5 ± 1.4
	N	7	11	12	5
Wk 10	**% ± SEM**	92.7 ± 2.2	78.6 ± 3.6	90.1 ± 2.3	92.9 ± 2.7
	N	7	11	12	5
Wk 11	**% ± SEM**	89.8 ± 2.6	85.8 ± 2.8	92.7 ± 2.1	90.8 ± 3.2
	N	7	11	12	5

At the AM scan samples, the percentage of birds sitting declined sharply between wk 4 and 5 for all treatments (Table 14). The percentage of NE-treated birds sitting increased at wk 6 and 7 while the percentage of saline birds sitting at increased at wk 6. At wk 7, the percentage of saline and intact birds sitting decreased before remaining approximately constant while the percentage of NE-treated birds decreased after wk 7. There were main effects of age ($P < 0.0001$, $F_{2, 681} = 41.99$) for wk 4 to wk 6 and differences in treatment-age interactions ($P = 0.0002$, $F_{12, 1364} = 3.13$) for wk 7 to wk 11. Intact birds tended to sit less frequently than high dose ($P = 0.0828$) and low dose ($P = 0.08223$) birds at wk 7. High dose birds sat more frequently at wk 7 than wks 8 ($P = 0.0460$), 9 ($P = 0.0460$), 10 ($P < 0.0001$), and 11 ($P < 0.0001$). Low dose birds sat more frequently at wk 7 than wks 8 ($P = 0.0070$), 9 ($P < 0.001$), 10 ($P = 0.001$), and 11 ($P < 0.0001$).

Table 14. Average percentage ± SEM of birds sitting at the AM scan samples.

		High	Low	Saline	Intact
Wk 4	**% ± SEM**	24.1±3.9	30.6±3.9	25.9±3.5	28.9±5.0
	N	7	9	9	5
Wk 5	**% ± SEM**	10.2±2.3	8.0±2.0	9.1±2.1	8.1±2.3
	N	7	9	9	5
Wk 6	**% ± SEM**	14.5±2.9	9.3±1.9	16.2±2.4	8.8±2.6
	N	7	9	9	5
Wk 7	**% ± SEM**	15.5±4.2	16.3±3.0	5.7±1.6	1.7±1.2
	N	7	12	12	5
Wk 8	**% ± SEM**	5.5±2.3	7.6±2.2	6.3±1.9	1.7±1.2
	N	7	11	12	5
Wk 9	**% ± SEM**	5.5±2.5	2.8±1.1	7.3±1.8	1.3±0.9
	N	7	11	12	5
Wk 10	**% ± SEM**	2.1±1.2	7.0±1.8	5.7±1.8	1.3±1.3
	N	7	11	12	5
Wk 11	**% ± SEM**	0.0±0.0	4.7±1.5	3.6±1.3	4.6±2.0
	N	7	11	12	5

At the PM scan samples, the percentage of birds sitting generally decreased until wk 8 before remaining constant for all treatments although the percentage of intact birds sitting increased at wk 7 (Table 15). There were main effects of age for wk 4 to wk 6 ($P < 0.0001$, $F_{2, 688} = 21.22$) and for wk 7 to wk 11 ($P < 0.0001$, $F_{4, 1203} = 8.62$). High dose birds sat more at wk 4 than wk 6 ($P < 0.0001$) and tended to sit more at wk 4 than wk 5 ($P = 0.0754$). Low dose birds sat more at wk 4 than wk 6 ($P = 0.0211$) and less at wk 8 than wk 7 ($P = 0.0402$) and wk 9 ($P = 0.0383$). Intact birds sat more at wk 4 than wk 5 ($P = 0.0108$) and wk 6 ($P = 0.0006$). Intact birds also sat more at wk 7 than wks 8 ($P < 0.0001$), 10 ($P = 0.0020$), and 11 ($P = 0.0109$) and tended to sit more at wk 7 than wk 9 ($P = 0.0556$).

Table 15. Average percentage ± SEM of birds sitting at the PM scan samples.

		High	Low	Saline	Intact
Wk 4	**% ± SEM**	44.8±4.5	31.0±3.5	30.2±4.1	45.6±4.6
	N	7	9	9	5
Wk 5	**% ± SEM**	33.5±3.5	28.0±3.1	23.9±2.7	27.5±4.3
	N	7	9	9	5
Wk 6	**% ± SEM**	22.6±3.2	18.7±2.7	21.5±2.8	22.9±3.9
	N	7	9	9	5
Wk 7	**% ± SEM**	17.3±3.8	19.1±2.9	13.0±2.9	25.8±4.5
	N	7	12	12	5
Wk 8	**% ± SEM**	9.4±3.1	9.5±2.1	8.3±2.1	3.3±1.6
	N	7	11	12	5
Wk 9	**% ± SEM**	7.8±2.5	11.4±2.8	6.8±1.8	2.5±1.4
	N	7	11	12	5
Wk 10	**% ± SEM**	7.3±1.2	12.3±1.8	9.9±1.8	7.1±1.3
	N	7	11	12	5
Wk 11	**% ± SEM**	10.2±2.6	14.2±2.8	7.3±2.1	9.2±3.2
	N	7	11	12	5

At the AM scan samples, there were smaller percentages of low dose birds vigilant compared to high dose, saline, and intact birds as juveniles (Table 16). In maturity, the intact treatment demonstrated the lowest percentage of birds vigilant. There were main effects of age ($P = 0.0112$, $F_{2, 682} = 4.52$) for wk 4 to wk 6 and differences in treatment-age interactions ($P = 0.0359$, $F_{12, 1362} = 1.85$) for wk 7 to wk 11. High dose birds tended to be less vigilant at wk 4 than wk 6 ($P = 0.0533$). Low dose birds were less vigilant at wk 7 than wk 8 ($P = 0.0451$). Saline birds were more vigilant at wk 6 than wk 4 ($P = 0.0472$) and wk 5 ($P = 0.0397$) and at wk 7 than wk 10 (0.0149). Saline birds tended to be more vigilant at wk 7 than wk 11 ($P = 0.0781$).

Table 16. Average percentage ± SEM of birds vigilant at the AM scan samples.

		High	Low	Saline	Intact
Wk 4	**% ± SEM**	19.3±2.5	16.0±2.1	23.1±2.6	24.8±3.8
	N	7	9	9	5
Wk 5	**% ± SEM**	23.7±2.8	18.3±2.3	22.9±2.7	32.3±4.2
	N	7	9	9	5
Wk 6	**% ± SEM**	28.7±2.8	20.8±2.8	31.6±3.1	25.8±3.7
	N	7	9	9	5
Wk 7	**% ± SEM**	21.1±3.9	13.5±2.4	27.6±3.7	14.6±3.4
	N	7	12	12	5
Wk 8	**% ± SEM**	20.1±3.8	24.2±3.7	19.3±2.6	14.6±3.5
	N	7	11	12	5
Wk 9	**% ± SEM**	21.4±4.0	18.7±3.0	21.4±3.3	15.4±4.1
	N	7	11	12	5
Wk 10	**% ± SEM**	16.4±3.1	21.8±3.2	15.1±2.7	10.8±3.1
	N	7	11	12	5
Wk 11	**% ± SEM**	12.2±2.9	17.4±3.0	17.2±3.0	18.3±4.3
	N	7	11	12	5

At the PM scan samples, saline birds were more vigilant than other treatments around the onset of sexual maturity while high dose birds were less vigilant in later weeks (Table 17). Vigilance decreased for all treatments at wk 11. There were differences in treatment-age interactions ($P = 0.0457$, $F_{6, 689} = 2.15$) for wk 4 to 6 and trends in treatment-age interactions ($P = 0.0811$, $F_{12, 1200} = 1.62$) and main effects of age ($P < 0.0001$, $F_{4, 1205} = 6.18$) for wk 7 to wk 11. Saline birds were less vigilant at wk 5 than wk 6 ($P = 0.0311$) and more vigilant at wk 8 than wks 9 ($P = 0.0257$), 10 ($P < 0.0001$), and 11 ($P < 0.0001$). High dose birds were less vigilant at wk 11 than wk 7 ($P = 0.0267$) and wk 9 ($P = 0.0374$).

Table 17. Average percentage ± SEM of birds vigilant at the PM scan samples.

		High	Low	Saline	Intact
Wk 4	**% ± SEM**	22.3±3.1	20.1±2.8	27.9±2.6	29.0±4.8
	N	7	9	9	5
Wk 5	**% ± SEM**	19.5±2.4	23.5±2.6	20.5±2.5	25.9±4.4
	N	7	9	9	5
Wk 6	**% ± SEM**	24.2±3.5	20.5±2.7	30.2±3.1	18.5±3.3
	N	7	9	9	5
Wk 7	**% ± SEM**	25.9±4.1	20.3±3.2	29.2±3.7	23.3±4.9
	N	7	12	12	5
Wk 8	**% ± SEM**	21.9±4.1	30.7±3.8	38.0±4.0	24.2±4.6
	N	7	11	12	5
Wk 9	**% ± SEM**	24.2±3.8	21.6±3.3	20.8±3.2	17.5±4.2
	N	7	11	12	5
Wk 10	**% ± SEM**	18.2±3.4	25.6±3.6	19.3±3.2	24.2±5.0
	N	7	11	12	5
Wk 11	**% ± SEM**	10.7±3.0	20.5±3.3	19.3±3.2	14.6±3.3
	N	7	11	12	5

At the AM scan samples, the percentage of birds pecking at the cage generally increased for all treatments (Table 18). High dose and saline birds pecked at the cage less at wk 7 while intact birds pecked at the cage more at wk 9. Mature low dose birds pecked at the cage more than other treatments. There were no main effects or treatment-age interactions ($P = 0.2135$, $F_{6, 682} = 1.4$) for wk 4 to wk. There were main age tendencies ($P = 0.0726$, $F_{4, 1366} = 2.15$) for wk 7 to wk 11 but no trends in treatment and wk comparisons following Sidak adjustments.

Table 18. Average percentage ± SEM of birds pecking the cage at the AM scan samples.

		High	Low	Saline	Intact
Wk 4	**% ± SEM**	1.3±0.7	2.1±0.7	2.2±0.8	0.5±0.5
	N	7	9	9	5
Wk 5	**% ± SEM**	1.9±0.7	1.4±0.5	1.7±1.0	3.5±1.4
	N	7	9	9	5
Wk 6	**% ± SEM**	2.4±1.0	2.6±0.8	1.3±0.6	4.2±1.4
	N	7	9	9	5
Wk 7	**% ± SEM**	0.0±0.0	5.7±1.8	1.6±0.9	3.3±1.9
	N	7	12	12	5
Wk 8	**% ± SEM**	1.8±1.1	5.1±1.6	4.7±1.5	4.6±2.2
	N	7	11	12	5
Wk 9	**% ± SEM**	4.7±1.8	6.1±1.7	1.0±0.7	3.3±1.9
	N	7	11	12	5
Wk 10	**% ± SEM**	4.7±1.8	5.5±1.7	4.7±1.5	9.2±3.5
	N	7	11	12	5
Wk 11	**% ± SEM**	5.5±2.3	4.2±1.4	3.1±1.2	1.3±1.3
	N	7	11	12	5

At the PM scan samples, percentage of birds pecking at the cage generally increased over time with intact birds exhibiting a decrease of pecking at wk 7 and a sharp increase in pecking at wk 9. High dose birds pecked at the cage less at wk 8 (Table 19). There were main effects of age (P = 0.002, $F_{2, 680} = 6.25$) for wk 4 to wk 6 and differences in treatment-age interactions (P = 0.0314, $F_{12, 1216} = 1.89$) for wk 7 to wk 11. High dose birds pecked at the cage less than intact birds at wk 9 (P = 0.0476) and more than low dose birds at wk 11 (P = 0.0383). High dose birds pecked at the cage more at wk 11 than wks 7 (P = 0.0159), 8 (P = 0.0050), and 9 (P = 0.0335). Low dose birds pecked at the cage less at wk 4 than wk 6 (P = 0.0241) and tended to peck the cage less at wk 5 than wk 6 (P = 0.0871). Saline birds tended to peck at the cage less at wk 5 than wk 6 (P = 0.0697). Intact birds tended to peck less at the cage at wk 7 than wk 11 (P = 0.0818).

Table 19. Average percentage ± SEM of birds pecking the cage at the PM scan samples.

		High	Low	Saline	Intact
Wk 4	**% ± SEM**	1.7±0.9	0.5±0.3	1.5±0.6	1.5±0.8
	N	7	9	9	5
Wk 5	**% ± SEM**	1.0±0.6	0.9±0.5	0.7±0.5	0.4±0.4
	N	7	9	9	5
Wk 6	**% ± SEM**	1.7±0.7	3.0±1.0	2.9±1.0	2.9±1.3
	N	7	9	9	5
Wk 7	**% ± SEM**	0.9±0.9	3.1±1.2	2.6±1.1	0.0±0.0
	N	7	12	12	5
Wk 8	**% ± SEM**	0.0±0.0	2.3±1.1	2.6±1.4	3.3±1.9
	N	7	11	12	5
Wk 9	**% ± SEM**	2.3±1.3	4.9±1.6	2.1±1.0	7.9±2.8
	N	7	11	12	5
Wk 10	**% ± SEM**	5.5±2.3	6.3±1.8	3.6±1.3	5.4±2.3
	N	7	11	12	5
Wk 11	**% ± SEM**	7.8±2.8	2.3±1.1	3.6±1.3	7.9±3.3
	N	7	11	12	5

At the AM scan samples, percentages of birds scratching the floor were highly variable with no birds scratching the floor at wk 4 and intact birds scratching the floor only at wk 11 (Table 20).Other treatments alternated between not scratching the floor and scratching the floor with low frequencies every other wk. There were no main effects or treatment-age interactions for wk 4 to wk 6 (P = 0.7006, $F_{6, 682} = 0.64$) or for wk 7 to wk 11 (P = 0.3119, $F_{12, 1370} = 1.15$).

Table 20. Average percentage ± SEM of birds scratching the floor at the AM scan samples.

		High	Low	Saline	Intact
Wk 4	**% ± SEM**	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0
	N	7	9	9	5
Wk 5	**% ± SEM**	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0
	N	7	9	9	5
Wk 6	**% ± SEM**	0.4±0.4	0.0±0.0	0.2±0.2	0.0±0.0
	N	7	9	9	5
Wk 7	**% ± SEM**	0.0±0.0	0.3±0.3	0.0±0.0	0.0±0.0
	N	7	12	12	5
Wk 8	**% ± SEM**	0.0±0.0	0.0±0.0	0.5±0.5	0.0±0.0
	N	7	11	12	5
Wk 9	**% ± SEM**	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0
	N	7	11	12	5
Wk 10	**% ± SEM**	0.5±0.5	0.0±0.0	0.5±0.5	1.3±1.3
	N	7	11	12	5
Wk 11	**% ± SEM**	0.0±0.0	1.7±1.0	0.5±0.5	0.0±0.0
	N	7	11	12	5

At the PM scan samples, percentages of birds scratching the floor were variable (Table 21). Low dose and saline birds scratched the floor throughout development. High dose birds only scratched the floor at wk 5. Intact birds did not scratch the floor at all. There were no main effects or treatment-age interactions ($P = 0.1899$, $F_{6, 686} = 1.46$) for wk 4 to wk 6, but there were main age tendencies ($P = 0.0755$, $F_{4, 1211} = 2.13$) for wk 7 to wk 11. Low dose birds scratched the floor more at wk 8 than wk 7 ($P = 0.0060$) and wk 11 ($P = 0.0090$). Low dose birds tended to scratch the floor more at wk 8 than wk 9 ($P = 0.0735$). Saline birds tended to scratch the floor more at wk 8 than wk 7 ($P = 0.0892$) and wk 10 ($P = 0.0892$).

Table 21. Average percentage ± SEM of birds scratching the floor at the PM scan samples.

		High	Low	Saline	Intact
Wk 4	**% ± SEM**	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0
	N	7	9	9	5
Wk 5	**% ± SEM**	0.3±0.3	0.2±0.2	0.3±0.3	0.0±0.0
	N	7	9	9	5
Wk 6	**% ± SEM**	0.0±0.0	1.0±0.5	0.2±0.2	0.0±0.0
	N	7	9	9	5
Wk 7	**% ± SEM**	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0
	N	7	12	12	5
Wk 8	**% ± SEM**	0.0±0.0	2.1±1.0	1.6±0.9	0.0±0.0
	N	7	11	12	5
Wk 9	**% ± SEM**	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0
	N	7	11	12	5
Wk 10	**% ± SEM**	0.0±0.0	0.9±0.7	0.0±0.0	0.0±0.0
	N	7	11	12	5
Wk 11	**% ± SEM**	0.0±0.0	0.0±0.0	0.5±0.5	0.0±0.0
	N	7	11	12	5

At the AM scan samples, preening decreased over time with juvenile high dose birds preening more than other treatments of the same age and mature low dose birds preening more than other treatments of the same age (Table 22). There were main treatment tendencies ($P = 0.0831$, $F_{3, 25} = 2.49$) for wk 4 to wk 6 and main effects of age ($P < 0.0001$, $F_{4, 1361} = 6.67$) for wk 7 to wk 11. High dose birds preened more than saline birds at wk 5 ($P = 0.0372$). High dose birds preened more at wk 7 than wk 9 ($P = 0.0169$), 10 ($P = 0.0080$), and 11 ($P = 0.02178$). Intact birds preened more at wk 7 than wk 10 ($P = 0.0149$).

Table 22. Average percentage ± SEM of birds preening at the AM scan samples.

		High	Low	Saline	Intact
Wk 4	**% ± SEM**	17.2±2.5	19.6±2.1	18.0±2.2	18.0±2.7
	N	7	9	9	5
Wk 5	**% ± SEM**	20.6±2.7	16.9±2.0	12.4±1.7	15.7±2.6
	N	7	9	9	5
Wk 6	**% ± SEM**	18.5±2.7	16.3±2.0	12.9±1.6	16.8±2.7
	N	7	9	9	5
Wk 7	**% ± SEM**	20.5±4.2	12.0±2.2	12.5±2.8	16.7±4.1
	N	7	12	12	5
Wk 8	**% ± SEM**	7.8±2.3	10.2±2.6	10.4±2.2	10.0±3.5
	N	7	11	12	5
Wk 9	**% ± SEM**	10.7±2.7	13.3±2.6	8.3±1.9	7.9±2.9
	N	7	11	12	5
Wk 10	**% ± SEM**	7.0±2.1	10.8±2.3	8.3±2.1	1.7±1.2
	N	7	11	12	5
Wk 11	**% ± SEM**	8.1±2.5	6.1±1.9	6.8±1.8	6.7±3.1
	N	7	11	12	5

At the PM scan samples, preening declined over time with juvenile NE-treated birds exhibiting more preening behavior than control birds (Table 23). There were differences in treatment-age interactions ($P = 0.0291$, $F_{6, 689} = 2.36$) for wk 4 to wk 6 and main effects of age ($P = 0.0058$, $F_{4, 1210} = 3.64$) for wk 7 to wk 11. High dose birds preened less frequently than low dose birds at wk 4 ($P = 0.0042$) and tended to preen less frequently than low dose birds at wk 5 ($P = 0.0972$). Low dose birds preened more at wk 4 than wk 5 ($P = 0.0359$). High dose birds preened more at wk 7 than wk 10 ($p = 0.0286$) and tended to preen more at wk 7 than wk 11 ($P = 0.0697$).

Table 23. Average percentage ± SEM of birds preening at the PM scan samples.

		High	Low	Saline	Intact
Wk 4	**% ± SEM**	16.5±2.4	23.4±2.3	13.1±1.7	16.2±2.5
	N	7	9	9	5
Wk 5	**% ± SEM**	23.3±2.3	16.1±2.1	16.4±2.0	18.3±3.3
	N	7	9	9	5
Wk 6	**% ± SEM**	17.2±1.9	21.8±2.3	16.4±2.0	16.6±2.5
	N	7	9	9	5
Wk 7	**% ± SEM**	16.7±3.8	12.5±2.3	12.0±2.3	11.3±3.4
	N	7	12	12	5
Wk 8	**% ± SEM**	7.8±2.2	13.1±2.7	13.0±2.3	15.4±3.9
	N	7	11	12	5
Wk 9	**% ± SEM**	11.7±2.8	15.5±2.7	12.5±2.2	6.2±2.4
	N	7	11	12	5
Wk 10	**% ± SEM**	4.9±2.1	10.0±2.3	9.4±2.0	10.0±3.5
	N	7	11	12	5
Wk 11	**% ± SEM**	6.0±2.0	7.2±1.9	9.4±2.1	5.4±2.4
	N	7	11	12	5

At the AM scan samples, juvenile NE-treated birds exhibited gentle feather pecking more than control birds with an increase in gentle feather pecking at wk 7 in high dose birds (Table 24). After wk 8, saline birds demonstrated similar or greater gentle feather pecking frequencies compared to NE-treated birds. Mature intact birds did not exhibit gentle feather pecking. There were main age trends ($P = 0.0911$, $F_{2, 682} = 2.4$) for wk 4 to wk 6 and differences in treatment-age interactions ($P = 0.0419$, $F_{12, 1369} = 1.81$) for wk 7 to wk 11. High dose birds exhibited more gentle feather pecking than low dose birds at wk 4 ($P = 0.0006$) and more gentle feather pecking than saline ($P = 0.0012$) and intact ($P = 0.0084$) birds at wk

7. High dose birds exhibited more gentle feather pecking at wk 7 than wks 8, 9, and 11 (P = 0.0070).

Table 24. Average percentage ± SEM of birds gently feather pecking at the AM scan samples.

		High	Low	Saline	Intact
Wk 4	**% ± SEM**	1.2±0.6	0.2±0.2	1.4±0.5	1.3±0.8
	N	7	9	9	5
Wk 5	**% ± SEM**	0.7±0.5	0.5±0.3	0.2±0.2	0.0±0.0
	N	7	9	9	5
Wk 6	**% ± SEM**	0.7±0.5	1.3±0.5	0.5±0.3	0.4±0.4
	N	7	9	9	5
Wk 7	**% ± SEM**	2.4±1.4	0.0±0.0	0.0±0.0	0.0±0.0
	N	7	12	12	5
Wk 8	**% ± SEM**	0.0±0.0	0.6±0.6	0.5±0.5	0.0±0.0
	N	7	11	12	5
Wk 9	**% ± SEM**	0.0±0.0	0.0±0.0	1.0±0.7	0.0±0.0
	N	7	11	12	5
Wk 10	**% ± SEM**	0.5±0.5	0.0±0.0	0.5±0.5	0.0±0.0
	N	7	11	12	5
Wk 11	**% ± SEM**	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0
	N	7	11	12	5

At the PM scan samples, gentle feather pecking was highly variable but seen in juvenile birds more than mature birds. Low dose and saline birds demonstrated some gentle feather pecking at wk 10 (Table 25).There were no main effects or treatment-age interactions for wk 4 to wk 6 (P = 0.5267, $F_{6, 686} = 1.0$) or for wk 7 to wk 11 (P = 0.8229, $F_{12, 1203} = 0.62$).

Table 25. Average percentage ± SEM of birds gently feather pecking at the PM scan samples.

		High	Low	Saline	Intact
Wk 4	**% ± SEM**	0.4±0.4	0.9±0.5	1.1±0.5	0.0±0.0
	N	7	9	9	5
Wk 5	**% ± SEM**	1.0±0.6	1.6±0.7	0.7±0.4	0.5±0.5
	N	7	9	9	5
Wk 6	**% ± SEM**	1.1±0.8	0.0±0.0	0.6±0.4	0.0±0.0
	N	7	9	9	5
Wk 7	**% ± SEM**	0.0±0.0	0.0±0.0	0.5±0.5	0.0±0.0
	N	7	12	12	5
Wk 8	**% ± SEM**	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0
	N	7	11	12	5
Wk 9	**% ± SEM**	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0
	N	7	11	12	5
Wk 10	**% ± SEM**	0.0±0.0	1.1±0.8	0.5±0.5	0.0±0.0
	N	7	11	12	5
Wk 11	**% ± SEM**	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0
	N	7	11	12	5

At the AM scan samples, intact birds exhibited severe feather pecking at wk 4 but did not exhibit feather pecking in later wks (Table 26). Low dose and saline birds demonstrated some severe feather pecking at wk 5 and low dose birds demonstrated severe feather pecking at 8, but high dose birds did not exhibit any severe feather pecking. There were trends in treatment-age interactions ($P = 0.0514$, $F_{6, 682} = 2.1$) for wk 4 to wk 6 but no main effects or treatment-age interactions ($P = 0.7105$, $F_{12, 1370} = 0.74$) for wk 7 to wk 11. Low dose birds exhibited a greater severe feather pecking frequency than intact birds at wk 4 ($P = 0.0072$). Intact birds exhibited a greater severe feather pecking frequency than high dose ($P = 0.0119$), low dose, and saline (0.0066) birds at wk 4. Intact birds exhibited greater severe feather pecking frequencies at wk 4 then wk 5 and wk 6 ($P = 0.0131$ for both wks).

Table 26. Average percentage ± SEM of birds severely feather pecking at the AM scan samples.

		High	Low	Saline	Intact
Wk 4	**% ± SEM**	0.0±0.0	0.0±0.0	0.0±0.0	0.8±0.6
	N	7	9	9	5
Wk 5	**% ± SEM**	0.0±0.0	0.2±0.2	0.3±0.3	0.0±0.0
	N	7	9	9	5
Wk 6	**% ± SEM**	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0
	N	7	9	9	5
Wk 7	**% ± SEM**	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0
	N	7	12	12	5
Wk 8	**% ± SEM**	0.0±0.0	0.6±0.6	0.0±0.0	0.0±0.0
	N	7	11	12	5
Wk 9	**% ± SEM**	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0
	N	7	11	12	5
Wk 10	**% ± SEM**	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0
	N	7	11	12	5
Wk 11	**% ± SEM**	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0
	N	7	11	12	5

At the PM scan samples, there were no main effects or treatment-age interactions ($P = 0.1509$, $F_{6, 686} = 1.58$) for wk 4 to wk 6 but there were main effects of age ($P = 0.0455$, $F_{4, 1210} = 2.44$) for wk 7 to wk 11. However, there were no differences in comparisons following a Sidak correction.

Table 27. Average percentage ± SEM of birds severely feather pecking at the PM scan samples.

		High	Low	Saline	Intact
Wk 4	**% ± SEM**	0.0±0.0	0.9±0.7	0.3±0.3	0.8±0.6
	N	7	9	9	5
Wk 5	**% ± SEM**	1.0±0.5	0.7±0.4	0.6±0.4	2.4±1.0
	N	7	9	9	5
Wk 6	**% ± SEM**	1.4±0.7	0.2±0.2	0.7±0.4	0.0±0.0
	N	7	9	9	5
Wk 7	**% ± SEM**	0.9±0.9	0.5±0.5	0.5±0.5	2.1±1.5
	N	7	12	12	5
Wk 8	**% ± SEM**	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0
	N	7	11	12	5
Wk 9	**% ± SEM**	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0
	N	7	11	12	5
Wk 10	**% ± SEM**	0.0±0.0	0.4±0.4	0.0±0.0	1.3±1.3
	N	7	11	12	5
Wk 11	**% ± SEM**	0.0±0.0	0.0±0.0	0.5±0.5	0.8±0.8
	N	7	11	12	5

At the AM scan samples, threatening behavior was limited among all treatments to the juvenile period with control birds displaying greater frequencies of threats if threats occurred in the treatment for the respective wk (Table 28). There were no main effects or differences in treatment-age interactions ($P = 0.1484$, $F_{6,682} = 1.59$) for wk 4 to wk 6 and no birds exhibited threatening behavior from wk 7 to wk 11.

Table 28. Average percentage ± SEM of birds showing threatening behavior at the AM scan samples.

		High	Low	Saline	Intact
Wk 4	**% ± SEM**	0.0±0.0	0.2±0.2	0.9±0.9	0.0±0.0
	N	7	9	9	5
Wk 5	**% ± SEM**	0.0±0.0	0.0±0.0	1.4±0.8	0.0±0.0
	N	7	9	9	5
Wk 6	**% ± SEM**	0.0±0.0	0.8±0.6	0.0±0.0	1.5±1.1
	N	7	9	9	5
Wk 7	**% ± SEM**	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0
	N	7	12	12	5
Wk 8	**% ± SEM**	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0
	N	7	11	12	5
Wk 9	**% ± SEM**	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0
	N	7	11	12	5
Wk 10	**% ± SEM**	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0
	N	7	11	12	5
Wk 11	**% ± SEM**	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0
	N	7	11	12	5

At the PM scan samples, low dose birds exhibited the greatest frequency of threats as juveniles compared to other treatments and intact birds exhibited no threatening behavior (Table 29). There were main effects of age ($P = 0.0139$, $F_{2, 683} = 4.3$) for wk 4 to wk 6 and no

main effects or treatment-age interactions (P = 0.4188, $F_{12, 1221}$ = 1.03) for wk 7 to wk 11. Low dose birds at wk 6 displayed a greater frequency of threats than wk 4 and wk 5 (P = 0.0143).

Table 29. Average percentage ± SEM of birds showing threatening behavior at the PM scan samples.

		High	Low	Saline	Intact
Wk 4	**% ± SEM**	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0
	N	7	9	9	5
Wk 5	**% ± SEM**	0.0±0.0	0.0±0.0	0.2±0.2	0.0±0.0
	N	7	9	9	5
Wk 6	**% ± SEM**	0.7±0.7	1.4±0.8	0.8±0.5	0.0±0.0
	N	7	9	9	5
Wk 7	**% ± SEM**	0.0±0.0	1.0±0.7	0.0±0.0	0.0±0.0
	N	7	12	12	5
Wk 8	**% ± SEM**	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0
	N	7	11	12	5
Wk 9	**% ± SEM**	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0
	N	7	11	12	5
Wk 10	**% ± SEM**	0.0±0.0	0.0±0.0	0.5±0.5	0.0±0.0
	N	7	11	12	5
Wk 11	**% ± SEM**	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0
	N	7	11	12	5

At the AM scan samples, aggressive pecking behavior increased in control birds until wk 6, peaked at wk 6 for NE-treated birds, then was absent after wk 6 with the exception of low dose birds displaying aggressive pecking at wk 8 (Table 30). High dose birds aggressively pecked more frequently than low dose birds. There were main effects of age (P = 0.0045, $F_{2, 682}$ = 5.44) for wk 4 to wk 6 but no main effects or treatment-age interactions (P = 0.7105, $F_{12, 1370}$ = 0.74) for wk 7 to wk 11. Intact birds tended to display a greater frequency of aggressive pecking at wk 6 than wk 4 (P = 0.0865).

Table 30. Average percentage ± SEM of birds showing aggressive pecking behavior at the AM scan samples.

		High	Low	Saline	Intact
Wk 4	**% ± SEM**	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0
	N	7	9	9	5
Wk 5	**% ± SEM**	0.0±0.0	0.0±0.0	0.2±0.2	0.4±0.4
	N	7	9	9	5
Wk 6	**% ± SEM**	0.7±0.5	0.3±0.3	0.5±0.3	1.0±0.7
	N	7	9	9	5
Wk 7	**% ± SEM**	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0
	N	7	12	12	5
Wk 8	**% ± SEM**	0.0±0.0	0.6±0.6	0.0±0.0	0.0±0.0
	N	7	11	12	5
Wk 9	**% ± SEM**	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0
	N	7	11	12	5
Wk 10	**% ± SEM**	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0
	N	7	11	12	5
Wk 11	**% ± SEM**	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0
	N	7	11	12	5

At the PM scan samples, low dose birds exhibited greater frequencies of aggressive pecking behavior compared to other treatments at wks 5, 7, and 8, and high dose birds exhibited a greater frequency of aggressive pecking behavior at wk 6 (Table 31). Only low dose birds displayed aggressive pecking behavior in maturity. There were differences in treatment-age interactions ($P = 0.0322$, $F_{6, 689} = 2.31$) for wk 4 to wk 6 but no main effects or differences in treatment-age interactions ($P = 0.9645$, $F_{12, 1048} = 0.40$) for wk 7 to wk 11. High dose birds aggressively pecked more at wk 6 than wk 4 and wk 5 ($P = 0.0311$ for both wks).

Table 31. Average percentage ± SEM of birds showing aggressive pecking behavior at the PM scan samples.

		High	Low	Saline	Intact
Wk 4	**% ± SEM**	0.0±0.0	0.0±0.0	0.2±0.2	0.0±0.0
	N	7	9	9	5
Wk 5	**% ± SEM**	0.0±0.0	0.7±0.5	0.2±0.2	0.0±0.0
	N	7	9	9	5
Wk 6	**% ± SEM**	1.1±0.6	0.0±0.0	0.0±0.0	0.5±0.5
	N	7	9	9	5
Wk 7	**% ± SEM**	0.0±0.0	0.5±0.5	0.0±0.0	0.0±0.0
	N	7	12	12	5
Wk 8	**% ± SEM**	0.0±0.0	0.6±0.6	0.0±0.0	0.0±0.0
	N	7	11	12	5
Wk 9	**% ± SEM**	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0
	N	7	11	12	5
Wk 10	**% ± SEM**	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0
	N	7	11	12	5
Wk 11	**% ± SEM**	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0
	N	7	11	12	5

Discussion

Our research sought to determine the effects of elevated *in ovo* NE on productivity and survival-related behaviors. High and low dose NE -treated birds experienced decreased average body weights until early sexual maturity compared to control birds. Norepinephrine birds also experienced dose-dependent delayed egg production compared to saline birds at the observed ages. Norepinephrine treatment had dose- and age-dependent long-term impacts on survival-related behaviors. Mature high dose birds ate more frequently while juvenile low dose birds drank more frequently compared to control birds. High dose birds foraged less frequently and were more inactive as juveniles. Low dose birds walked less compared to control birds. After rehoming, the NE-treated birds displayed notable variations in behaviors that have traditionally been accepted as key to assessing well-being, such as inactivity and foraging, as well as multiple other patterns of behavior. At week 7 there were high dose increases and low dose, saline, and intact decreases in gentle feather pecking, intact increases in severe feather pecking, decreases in aggressive pecking across all treatments, indicating altered social interactions. There were also decreases in low dose and increases in high dose preening and decreases in vigilance in all treatments. Throughout the study, juvenile low dose birds and mature high dose birds were less vigilant, high and low dose birds preened more, low dose birds pecked at the cage more, and low dose birds exhibited more threats,. Overall, it is evident from our results that elevated embryonic NE alters productivity and survival-related behaviors, and stress-coping mechanisms in a dose- and age-dependent manner throughout ontogenesis.

Norepinephrine and interacting monoamines and metabolites including homovanillic acid (HVA), 3-methoxy-4-hydroxyphenylglycol (MHPG), epinephrine (EP), dopamine (DA), and dihydroxyphenylacetic acid (DHPA) impact consumption behaviors, changing weights and activity levels in avian and mammalian species (Lichtenstein et al., 1984; Fruhstorfer et al., 1989; Denbow and Sheppard, 1993). We found that embryonic NE increases feeding frequency in mature high dose birds and drinking frequency in juvenile low dose birds as well as inactivity in high dose birds Walking was reduced in both high and low dose birds. These data demonstrate that embryonic NE produces similar behavioral effects as acutely-elevated NE but that differences in behavioral expression are variable at different stages of ontogeny. Previous work suggests that the CNS site of NE binding to α_2-adrenergic receptors dictates effects on feeding behavior (Denbow and Sheppard, 1993). In chicks, increased feeding behavior is associated with elevated NE, MHPG, and 5-HT levels in the HYP with consistently high NE and MHPG levels and decreasing 5-HT levels after 30 minutes of feeding (Tachibana et al., 2001). Injections of 33, 67, and 100 μg/10 μl of NE, EP, or DA to the lateral ventricles of the brain produce varying feeding and drinking patterns with dose-dependence as observed in our study (Denbow et al., 1981). While EP increases feed intake, DA has no effect on feed intake (Denbow et al., 1981). Norepinephrine can induce sedative and narcoleptic responses but increases feed intake in birds that do not demonstrate narcoleptic tendencies (Denbow et al., 1981; Denbow et al., 1983; Steinman et al., 1987; Denbow and Sheppard, 1993; Katayama et al., 2010). Norepinephrine injections to the lateral septal organ and anterior nucleus reticularis superior, pars dorsalis, and tractus

occipotomesencehpalicus decrease food intake (Denbow and Sheppard, 1993). However, NE injections to the preoptic hypothalamic region, the PVN, the ventromedial nucleus, and the medial septal nucleus increase food intake (Denbow and Sheppard, 1993). Injections to the basolateral regions of the preoptic area have a greater impact on increased drinking compared to medial regions (Denbow and Sheppard, 1993). These studies support our conclusions that elevated embryonic NE in Japanese quail produces dose-and age-dependent increases in consumption behavioral frequencies via impacts on hypothalamic networks.

Norepinephrine reduces activity in lower doses and causes narcolepsy in higher doses in chickens (Denbow et al., 1981; Denbow and Sheppard, 1993). In the context of our research, the high dose treatment of 10 µl of 0.05M NE caused increased inactivity but not full narcolepsy while the low dose treatment of 10 µl of 0.01M NE reduced walking frequencies. In humans, levels of DHPA, HVA, 5-HIAA, and MHPG in cerebrospinal fluid normally show predictable interactions, but cases of hyperinsomnia demonstrate a lack of correlation between MHPG and the other metabolites while cases of narcolepsy show a lack of correlation between DHPA and HVA and the other metabolites. These findings suggest a dysfunction of NE and DA pathways and subsequent metabolite interactions in individuals with narcolepsy (Faull et al., 1986). In neonatal chicks, intercerebroventricular injection of NE, clonidine (an α_2 agonist), or glucagon-like peptide-1 (GLP-1) results in narcoleptic effects related to α_2 receptor overstimulation (Denbow et al., 1981; Bungo et al., 1999; Bungo et al., 2001; Katayama et al., 2010). While we did not see a narcoleptic response in the NE-treated birds, our high dose treatment birds exhibited

reduced activity levels in early life and in response to rehoming stress and mature low dose birds walked less. Our research provides support of dose-dependent long-term effects of embryonic NE on activity levels in addition to the known dose-dependent short-term effects of post-hatch exogenous NE on activity, contributing to conclusions drawn by Denbow et al (1981) that the extent of activity depression is dependent on NE concentration as well as individual morphology, which NE may alter during ontogenesis.

We also found that NE treatment reduces weight gain in early life with low dose birds especially impacted. Although we measured feeding and drinking frequency, we did not measure feed and water intake directly. Smaller body weights in low dose birds at wk 3 may have been due to reduced feed intake. Juvenile high dose birds ate more frequently and were more inactive than other treatments, which could have also compensated for metabolic or feed nutrition efficiency impairments caused by elevated embryonic NE that led to the more pronounced reduced body weight averages in low dose birds. While the high dose birds ate more frequently, they may not have consumed as much feed as the larger body weight saline birds, but acute increases in NE have typically been associated with an increase in body weight related to increased feeding (Leibowitz et al., 1984). Repeated injection of NE into the PVN of rats stimulates feed intake regardless of restricted or ad libitum access to food, resulting in increased body weights over time (Leibowitz et al., 1984; Lichtenstein et al., 1984). In humans, changes in NE metabolism have been associated with eating disorders and dietary changes. Beta-2 polymorphisms that can ultimately result in obesity are linked to higher plasma NE levels, even before

excessive weight gain occurs (Masuo et al., 2005). Dietary weight loss decreases NE spillover, in turn decreasing sympathetic nervous system activity (Straznicky et al., 2005). Conversely, patients recovering from anorexia nervosa who are able to maintain weight for 6 months or longer demonstrate decreased plasma and cerebrospinal fluid levels of NE and MHPG compared to healthy controls suggesting that reduced noradrenergic activity might aid in long-term weight recovery rather than weight loss (Kaye et al., 1985).

While elevated NE has generally been associated with increased body weight related to diet, increased NE and interacting neuropeptides during early development negatively impact weight gain and growth according (Eriksen et al., 2003; Hayward and Wingfield, 2004). Our results corroborate preceding research as low dose birds weighed significantly less during early development, providing evidence that NE affects weight at various points in ontogenesis according to dosage. In humans, it has been shown that maternal stress leads to low birth weights and increased chance of preterm births (Rondo et al., 2003; Borders et al., 2007; Nkansah-Amankra et al., 2010). Psychologically stressed pregnant women exhibit elevated prenatal cortisol levels linked to lower fetal weights (Diego et al., 2006). In adult chickens, continuous administration of adrenocorticotropin hormone (ACTH), which stimulates NE and EP concentration, via an osmotic pump, results in elevated levels of plasma corticosterone with decreases in body weight and multiple organ weights (Valenta et al., 1986; Puvadolpirod and Thaxton, 2000). While we saw no difference in average body weights at one day of age or organ to body weight ratios at 11 wks of age between NE-treated and control chicks, we did see lower early life body weights in

the juvenile low dose NE birds. These data allow us to conclude that embryonic NE decreases weight gain at vital periods of development. Ultimately, reduced linear weight gain can impact long-term physical health (Walker et al., 2002).

Norepinephrine plays a role in reproduction with impacts on egg productivity via regulation of chicken Gonadotropin-Releasing Hormone-I (cGnRHI) and its release from perifused medial basal hypothalamic preoptic region explants in both male and female quail (Li et al., 1994; Millam et al., 1994; Millam et al., 1998). Norepinephrine stimulates Gonadotropin-Releasing Hormone-I which causes the pituitary gland to release Luteinizing Hormone (LH) and Follicle Stimulating Hormone (FSH) to mediate testicular and ovarian function, including egg production (Ottinger and Bakst, 1995). Once a female produces an egg, NE facilitates its release, inducing strong contractions then infundibulum, magnus, and isthmus relaxation to push the egg through the reproductive tract (Ottinger and Bakst, 1995). However, acute or temporary stress delays oviposition with laying hens retaining eggs up to several hours following the stressful event (Reynard and Savory, 1999). It has also been suggested that if NE injected into the PVN changes satiety and feeding-behaviors, it simultaneously alters reproductive behaviors that are controlled by the PVN (Denbow and Sheppard, 1993). Although we did not observe treatment or week differences in testes weights and ratios or mounting behavior during scan sampling, we did observe NE dose-dependent delayed egg production. Elevated NE during embryogenesis, potentially resulting from maternal stress, leads not to prolonged retention of an egg as in cases of short-term stress but to a lack of egg production altogether, contributing to an overall decrease in flock productivity and later age of

sexual maturity. We also observed differences in egg production between replicates related to later age of onset of sexual maturity in the second replicate. Further research can clarify factors that could have contributed to this variation and its effect on egg production. Overall, delayed and diminished egg production in birds with elevated *in ovo* NE may serve as an evolutionary strategy, preventing egg-related energy expenditures in offspring of stressed hens under conditions unconducive to survival, enhancing long-term reproductive fitness of individuals or offspring (Cody, 1966; Price, 1974; Sheriff and Love, 2013). Unpublished data obtained by Rudo et al, further support reduced egg lay as an evolutionary strategy to cope with stress with birds lacking environmental enrichment laying fewer but larger eggs compared to birds provided with environmental enrichment. Williams (2005) argues that study of the prolonged activity of hormones involved in reproductive processes will provide insight to mechanisms underlying the costs of egg production. Future lifetime studies of birds exposed to higher embryonic concentrations of NE will demonstrate whether delayed egg production extends lay as a bird ages and whether subsequent generations exhibit altered patterns of egg productivity.

Following rehoming, we observed changes in several survival-related behaviors of the quail including activity levels, foraging, and walking. Results lead to the conclusion that elevated *in ovo* NE alters stress-coping strategies. Numerous studies have provided evidence in both human and animal models that prenatal stress contributes to impaired behavioral and physiological stress-coping mechanisms and greater reactivity to stressful events (Beydoun and Saftlas, 2008; Van den Bergh et al., 2008). In humans, adult children of women who reported physically and/or

emotionally stressful pregnancies exhibit higher rates of PTSD, anxiety, ADHD, and depression (Watson et al., 1999; Brown et al., 2000; Talge et al., 2007; Kinsella and Monk, 2009). Prenatal stress leads to less exploratory behavior, reduced social behavior, impaired neuromotor behavior, and increased anxious and anhedonic behavior in rodents and primates (Schneider, 1992; Schneider and Coe, 1993; Morley-Fletcher et al., 2003; Lee et al., 2007). In birds, elevated corticosterone prior to egg incubation results in higher tonic immobility (TI) duration, increased fearfulness of humans, decreased imprinting, generally diminished competitive ability, and reduced ability to overcome an obstacle to access food (Rubolini et al., 2005; Janczak et al., 2006; Nordgreen et al., 2006; Janczak et al., 2007; Love and Williams, 2008; Chin et al., 2009). Elevated maternal corticosterone and late pre-hatch corticosterone also alter behavior and physiology (Henriksen et al., 2011). Scan sampling data in our study demonstrated drastic changes in several behaviors following rehoming in NE-treated birds suggesting altered stress-coping abilities. Norepinephrine-treated birds were more inactive and foraged less following rehoming. Type and complexity of behaviors including foraging and locomotion are important in assessing avian well-being in response to a stressor (Marıa et al., 2004). Changes in these survival-related behaviors due to elevated embryonic NE carry implications for animal welfare and industry management of breeding flocks.

Injection of NE into quail eggs at ED1 influences post-hatch productivity and behavior depending on NE dose and age at the time of behavioral expression. These changes may be rooted in physiological alterations of the HPA-axis, adrenergic receptor networks, and NE metabolism with effects on interacting neuropeptides.

Adrenergic activity has previously been implicated in weight gain and egg production (Verma and Walker, 1974; Sharma et al., 2001; Wang et al., 2005; Park et al., 2006). Beta-blockers produce weight gain in hypertensive patients while patients with certain α-receptor polymorphisms are more prone to weight gain when using α-blockers to treat affective disorders (Sharma et al., 2001; Wang et al., 2005; Park et al., 2006). In relation to egg productivity, use of phenoxybenzamine, an α- adrenergic receptor blocker, or propranolol, a β-adrenergic receptor blocker, inhibits NE induction of contractions to push an egg through the avian reproductive tract indicating α- and β-adrenergic receptor involvement (Verma and Walker, 1974). Adrenergic networks are also implicated in consumption patterns and activity levels. Clonidine increases feed intake in chickens suggesting that NE, which has a strong binding affinity for α_2-adrenergic receptors, mediates feeding behavior via α_2-adrenergic receptor networks (Choi et al., 1995). Canines with narcolepsy demonstrate an increased number of α-receptor binding sites in the LC (Fruhstorfer et al., 1989). Maternal stress alters HPA-activity and adrenergic formation in offspring, changing stress-coping abilities in later life. In offspring of stressed rats, there is a decrease in hippocampal synaptic density of adrenergic receptors and an increase in fetal Trp, 5-HT, and 5-HIAA (Peters, 1986; Hayashi et al., 1998). Dysregulation of adrenergic systems during or following development in rats increases vulnerability to stress, resulting in exaggerated HPA axis activity with effects on behavioral expression (Morilak et al., 2005). Elevated corticosterone in avian mothers results in increased HPA axis activity in offspring in response to restraint and handling stresses (Hayward and Wingfield, 2004). In our research, we

observed changes in weight gain, egg production, consumption behaviors, and activity levels reflective of behavioral expressions altered by interfering with normal adrenergic function. Although we saw reduced weight gain, we observed increased inactivity consistent with lower feed intake in animals with narcoleptic tendencies following NE administration. Considering NE's neurogenic effects in the context of prior studies, changes in behavioral expression of birds with elevated levels of embryonic NE could be explained by alteration of adrenergic density and binding affinity during early development.

Quail in our study demonstrated reduced weight, delayed egg production, increased consumption and decreased activity, and drastic patterns of change in survival-related behaviors following rehoming.Within the context of prior research, elevated *in ovo* NE, a known neurogenic compound, at the start of incubation may dictate these age-dependent differences in productivity and behavior via dose-dependent HPA-axis dysregulation and altered adrenergic network formation in areas of the central and peripheral nervous systems during embryonic as well as post-hatch development (Jhaveri et al., 2010). Although elevated embryonic NE does impact productivity and behaviors with some negative consequences for poultry well-being, ongoing research of the physiological mechanisms responsible for these long-term changes will provide a deeper understanding of NE's role in behavioral programming for positive states of well-being and improved poultry management. For instance, walking was frequently associated with pacing behavior among the quail in our study. However, low dose birds exhibited diminished frequencies of walking. Norepinephrine's behavioral programming in reduction of stereotypic and energy-

consuming walking could improve bird well-being in industry settings (Hill, 1983; Appleby, 1986). Further study of how elevated embryonic NE impacts lifetime and generational lay rates may also demonstrate a reduction of age effects on egg production and quality for improved yield in commercial layers (Joyner et al., 1987). Overall, poultry breeding flocks are constantly exposed to internal and environmental stressors which can lead to higher *in ovo* NE deposition with long-term effects on offspring behavior and neurophysiology. Ongoing research will provide additional information on how NE alters adrenergic formation during embryonic and post-hatch development, improving understandings related to *in ovo* NE manipulation for targeted behavioral programming in birds to better states of well-being and facilitate poultry management. However, our research shows that early embryonic exposure to the stress hormone NE affects productivity, survival-related behaviors, and stress-coping mechanisms in a dose- and age-dependent manner with implications for bird well-being and commercial management.

Chapter 4: A discussion of the effects of elevated embryonic norepinephrine on poultry behavior, physiology, and well-being

There is relatively little avian research on the role of elevated catecholamines during early embryogenesis in behavioral programming although the behavioral and physiological effects of maternal stress have been well documented in mammals (Groothuis et al., 2005; Weinstock, 2005; Beydoun and Saftlas, 2008; Kinsella and Monk, 2009). Our study assessed the impacts of elevated *in ovo* NE on cognition, productivity, survival-related behaviors, and stress-coping strategies to better define NE's role in ontogenesis in relation to poultry well-being. We found that elevated NE at the start of incubation had dose- and age-dependent effects on cognition, fear, and aggression as well as productivity and survival-related behaviors, especially in response to stress. Results provide evidence that maternal stress leading to elevated NE deposition in an egg increases or decreases cognitive ability and induces defensive or confronting stress-coping strategies according to age and embryonic NE concentrations with high dose birds generally showing more anxious behaviors. We also observed changes in normal survival-related behavioral patterns in response to rehoming stress, reductions in growth and egg productivity, increases in consumption behavioral frequencies, and decreases in activity levels in post-hatch offspring at different life stages based on NE doses.

Our data suggest that increased *in* ovo NE alters aspects of ontogenesis leading to multiple changes in fearful, aggressive, social, and survival-related behavioral expressions based in similar physiological mechanisms and impairment of stress-coping behaviors that can play role in weight gain and egg productivity. For instance, reduced foraging and feeding behavior and redirection of energy to prey-type behaviors can increase chances of survival in response to a predator, proving to be an effective fitness strategy (Schütz et al., 2001).

However, propensities for predator-based fear responses can be associated with physical impacts. Broiler chickens with higher tonic immobility durations have been shown to have higher levels of corticosterone, which positively interacts with NE, dramatically reducing growth rates compared to broilers with short TI durations (Wang et al., 2013). Similarly, in our study, trends in higher TI duration for low dose chicks coincided with significantly lower body weights among low dose juveniles at 3 wks of age, implying reduced feed intake or potential downstream effects of NE system alterations on biological compounds that regulate weight gain, such as corticosterone.

Performance on the memory test can also be related to weight. Maternal stress leading to elevated embryonic stress hormones and monoamines can result in low birthweights and reduced growth throughout development (Eriksen et al., 2003; Weinstock, 2005). However, children with pre-term births and low birthweights exhibit memory impairments and low hippocampal volumes (Isaacs et al., 2000). Low dose birds with reduced body weights at wk 3 could have exhibited lower hippocampal volumes in early life resulting in hindered performance when placed in a maze as juveniles. Physical activity additionally aides in memory abilities (Ruscheweyh et al., 2011). Reduced active behaviors in high dose birds may have contributed to diminished performance on the memory maze during maturity.

Hippocampal adrenergic system densities and binding affinities are also implicated in short-and long-term stress coping mechanisms with some level of plasticity mediated by NE concentrations during a stressful event (Tejani-Butt et al., 1994; Pandey et al., 1995). Anxiety responses in NE-treated birds included increased stationary bouts, increased defecation, and decreased foraging during an open field isolation stress test and increased inactivity, decreased foraging, and increased walking following rehoming compared to control birds. Impaired stress-coping abilities could be attributed to changes in hippocampal densities of

adrenergic networks associated with reduced hippocampal volume corresponding to low birth weights and subsequent memory impairment.

Genetic studies of Japanese quail have also shown that quantitative trait loci in the CJA01 chromosome regulate clutch size, body weight, feed intake, and higher TI duration at 8-9 days of age (Minvielle et al., 2005). However, associations between stress-coping abilities and weight and egg lay are not regulated by genes involved in TI duration given the distance between the loci. Age of the onset of egg production and the number of eggs laid is further associated with chromosome CJA06 (Minvielle et al., 2005). Decreased body weights, increased eating and drinking frequencies, increased TI duration as chicks, and delayed egg production observed in NE-treated Japanese quail in our own research suggest that elevated embryonic NE may play an epigenetic role in regulating CJA01 and CJA06.genetic expression.

Elevated embryonic NE influences cognitive, fearful, aggressive, social, and survival related behaviors as well as stress-coping mechanisms and productivity with real-world impacts on animal well-being and poultry management. Interactions between these outwardly visible phenotypes are consistent with changes in behavioral and physical expressions following alterations in adrenergic receptor binding affinity and density, HPA-axis formation, and NE metabolism. In both mammalian and avian species, maternal and incubation stresses cause increases in glucocorticoids and monoamines in the developing embryo with long-term impacts on development (Epple et al., 1997; Weinstock, 1997; 2005; Kinsella and Monk, 2009). Offspring are more prone to low birth weights and reduced weight gain, early mortality, blood pressure issues, and other health problems later in life (Diego et al., 2006; Borders et al., 2007; Nkansah-Amankra et al., 2010). Prenatally stressed offspring also show an increased incidence of affective disorders and impaired stress-coping abilities due to HPA-axis dysregulation, altered formation of adrenergic and serotonergic networks, elevated glucocorticoid and monoamine levels, and poor neurotransmitter degradation resulting from

impaired feedback mechanisms (Weinstock, 1997; Talge et al., 2007; Kinsella and Monk, 2009). Norepinephrine is neurogenic and naturally present in the quail chick embryo by ED3.5 and remains high in post-hatch offspring while waning in mature birds suggesting a vital role in early and continued developmental processes (Enemar et al., 1965; Markham et al., 2006; Jhaveri et al., 2010). Elevated levels of NE throughout juvenile and adolescent development and as well as in adulthood can increase or decrease α- and β-adrenergic receptor densities and binding affinities in the HIPP, HYP, and AMYG to offset increased adrenergic activity depending on whether the stress is acute or chronic, predictable or unpredictable, and mental or physical as well as the affected individual's demographics (Dimsdale et al., 1994; Tejani-Butt et al., 1994; Seo et al., 1999; Camp, 2015). It is therefore reasonable to propose the maternal stress leading to elevated *in ovo* NE alters adrenergic receptor formation at the beginning of embryogenesis in a dose-dependent manner with differing short- and long-term impacts on affective behavior and productivity throughout the course of development as seen in our study.

We can better understand how to manipulate embryonic NE levels to achieve positive states of well-being through behavioral programming in determining the precise physiological mechanisms underlying behavioral and productivity changes in poultry. Elevated *in ovo* NE in our research had some positive consequences for behavior. For instance, latencies to choose the first and the correct arms in a maze were smaller in low-dose birds and greater in high dose birds but juvenile high dose birds and mature low dose birds exhibited 83% and 100% correctness in the spatial memory test respectively. Laying hens in commercial open housing floor systems must learn to utilize nest boxes to reduce the number of dirty and broken eggs and increase egg collection efficiency (Appleby, 1984). In application to commercial settings, targeted manipulation of adrenergic systems during development to behaviorally program birds for improved cognitive ability at key ages may facilitate learning to use nest boxes resulting in easier management. Similarly, saline birds

walked and paced more frequently than NE-treated birds during scan sample observations. Stereotypic pacing behavior can be detrimental to bird well-being (Hill, 1983; Appleby, 1986). Behavioral programming via embryonic NE may help reduce pacing and other stereotypic behaviors in poultry flocks.

Norepinephrine is the primary neurotransmitter of the gut (see Appendix III for preliminary data on embryonic NE effects on ileal morphology). Hormones, including NE, facilitate cross-talk between the host and gut microbiota (Sperandio et al., 2003). Norepinephrine can be further implicated in shifts towards pathogenic bacteria under stressful conditions or in associations between central nervous system disorders including autism and anxiety and gastrointestinal disorders such as irritable bowel syndrome (Carabotti et al., 2015). Gut microbiota compositions and application of probiotics have previously been shown to alter affective behaviors both positively and negatively depending on bacterial strain ratios (Manco, 2012; Foster and Neufeld, 2013). As probiotic use becomes a more favorable option in improving gut health, gut-nervous system interactions via NE may be impacted. Depending on the type of bacteria administered, probiotics may serve as an effective vehicle for altering NE crosstalk during early development to positively program behavior and improve animal well-being.

Ultimately, behavioral alteration of stress-coping mechanisms in offspring in response to maternal stress and elevated embryonic NE could actually be evolutionarily adaptive. Boyce and Ellis (2005) propose that heightened reactivity and plasticity to stress in offspring may improve fitness under supportive conditions or prove detrimental under adverse conditions during development. Increased biological sensitivity to stress may also be countered by other mechanisms regulating physiological arousal. Findings from multiple studies corroborate this theory. Infants of mothers demonstrating depressive symptoms during and following pregnancy had increased motor and mental development at one year of age (Sandman et al., 2012). Similarly, avian studies show that under conditions of maternal stress,

high levels of androgens are deposited *in ovo*, altering aggressiveness, growth, and physiology to improve the offspring's ability to compete in the mother's stressful environment (Kaiser and Sachser, 2005). While growth was reduced in low dose birds in our study, high dose birds were more aggressive which could have been a manifestation of competitive strategies in asserting dominance and monopolizing resources. Unpublished data by Rudo et al, further demonstrates that quail receiving favorable environmental enrichment laid more but smaller eggs compared to quail that received no enrichment, suggesting adaptive egg production strategies to favorable versus unfavorable environments.. Delayed egg production in NE-treated quail could correspondingly be the result of an evolutionarily adaptive strategy conserving maternal energy in expected stressful environments or extending lifetime egg lay. Further research on the lifetime and generational effects of elevated NE-induced delay of egg production could demonstrate evolutionary strategies that improve fitness overall (Williams, 2005). Porges (1997) also suggests that evolution of the autonomic nervous system in response to affective experiences enhances phylogenetic emotional responses ultimately increasing fitness. Norepinephrine-treated birds may demonstrate nervous system changes that lead to amplified emotional reactivity to stress, including increased TI as chicks, anxiety behaviors in isolation, and variations in activity and foraging levels following rehoming. These differences in stress-coping abilities compared to control birds may have better prepared NE-treated birds for subsequent environmental and social interactions that demanded affective responses.

Behavioral effects of elevated embryonic NE carry implications for both domestic and wild fowl. In both the meat and egg industries, poultry are subject to a multitude of daily stresses. These stressors can lead to behavioral problems, poor animal well-being, and decreased productivity impacting industry profit (Hill, 1983; Jones and Hughes, 1986; Hemsworth and Barnett, 1989; Mills et al., 1990). Understanding how these stressors jeopardize bird well-being on behavioral and physiological levels is vital to ensuring that

appropriate measures are taken to prevent negative states and promote positive states in the birds. Poultry breeding flocks may be especially exposed to stressors that influence behavior and physiology due to high levels of physical aggression during mating, strict feeding regimens, and social stress (Rosales, 1994). According to our findings, elevated *in ovo* NE resulting from maternal stress significantly impacts the long-term productivity, behaviors, and stress-coping abilities of offspring. Our research thus highlights the need to reduce maternal stress to promote more positive states of well-being associated with improved stress-coping abilities within offspring as well as to meet industry goals of maximizing meat and egg output.

For wild birds, increased embryonic NE's effect on cognitive, affective, social, and survival-related behaviors as well as productivity may or may not be adaptive in the evolutionary scale depending on the environment. Low early life body weights, increased fearfulness, and inability to cope with stressors including decreased foraging and activity levels could prove detrimental to survival in extreme conditions (Lima, 1986; Hayward and Wingfield, 2004; Saino et al., 2005; Love et al., 2008). However, increased aggression in relation to resource competition and altered egg lay strategies may improve fitness (Weimerskirch, 1992; Mac Nally and Timewell, 2005; Williams, 2005). In addition to natural environmental stressors, urbanization has infringed on avian territories introducing new sources of stress including human contact, dietary changes, and lack of nesting space (Beissinger and Osborne, 1982). These stressors can negatively impact birds causing changes in stress physiology (Partecke et al., 2006). Our study provides further evidence that offspring of stressed avian mothers exhibit altered stress-coping mechanisms that may not prove beneficial in an unsupportive urban environment.

Overall, our research concludes that elevated embryonic NE impacts affective behaviors, stress-coping mechanisms, and productivity in a dose and age-dependent manner.

Altered behavioral expressions and productivity parameters show that increased *in ovo* NE influences a span of behavioral and physical attributes that may interact and magnify NE's effects at critical points throughout development. Placed in the context of prior research, changes in behavior and production suggest that elevated embryonic NE alters early adrenergic formation in both the central and peripheral nervous systems according to deposited concentrations at the start of incubation. Further work is needed to determine whether behavioral and physical effects are maladaptive or carry fitness and evolutionary benefits. However, our results currently show that while many behavioral and productivity changes are detrimental to bird well-being, some behaviors are positively altered. Our ongoing research on adrenergic receptor expression under conditions of elevated embryonic NE will provide further support of affected systems and offer insight into how physiological mechanisms can be targeted to beneficially program behavior in the future. In the present, findings carry implications for domestic and wild fowl, showing that elevated NE during embryogenesis changes behavior and physiology in ways that can jeopardize well-being and productivity by interfering with the birds' ability to thrive under various conditions and cope with inevitable stressors. Our study highlights the need to mitigate sources of stress and foster positive states of well-being through supportive environments to reduce the damaging effects of elevated embryonic NE. and improve welfare throughout the course of development.

Appendices

Appendix I – Behavioral Testing Ethogram

Table A1. Ethogram used for behavioral testing.

Behavior	Description
Stand	Upright on both feet with body raised from the ground
Sit	Stooped with legs bent and body touching the ground
Eat	Pecking at feed from trough
Drink	Pecking at water in automatic drinker
Forage	Moving with head to the ground, pecking with the intent to find food
Walk	Non-productive movement within the space
Inactive	Relaxed but awake and remaining in one place
Vigilant	Alert and observant of surroundings
Pecking at Cage	Using beak to strike the surroundings
Preening	Using beak to straighten and clean own feathers
Scratching floor	Feet and talons moved across the floor as if in an attempt to shift substrate
Gentle Feather Pecking	Pecking at a conspecific's body feathers in a non-injurious and social or investigative manner
Severe Feather Pecking	Pecking at a conspecific's body feathers in a injurious manner sometimes resulting in the feather being plucked from the conspecific
Aggressive pecking	Injurious pecking directed at a conspecific's head or neck
Threat	Standing upright with legs extended and hackles raised facing a submissive and cowering bird
Crouched	Cowering close to the ground in one place with feathers pulled close to body and little other movement
Erect	Body raised with head extended looking around
Jumping/flight	Attempts to remove feet from contact with ground while flapping wings

Appendix II – Scan Sample Data

Table A2. Average percentage of birds eating at the AM scan samples.

		High	Low	Saline	Intact
Week 4	**% ± SEM**	5.8±1.4	6.6±1.6	6.7±1.5	4.0±1.4
	N	7	9	9	5
Week 5	**% ± SEM**	8.7±1.7	10.7±1.7	5.6±1.3	5.7±1.7
	N	7	9	9	5
Week 6	**% ± SEM**	8.8±2.1	8.8±1.6	8.1±1.5	7.7±2.1
	N	7	9	9	5
Week 7	**% ± SEM**	8.6±2.5	9.7±2.3	6.3±1.7	8.3±2.5
	N	7	12	12	5
Week 8	**% ± SEM**	7.3±2.4	8.5±2.3	5.7±1.8	8.7±2.7
	N	7	11	12	5
Week 9	**% ± SEM**	9.6±2.7	4.0±1.5	5.7±2.1	7.5±2.7
	N	7	11	12	5
Week 10	**% ± SEM**	15.1±3.4	10.2±2.1	4.2±1.4	4.2±1.8
	N	7	11	12	5
Week 11	**% ± SEM**	14.6±3.1	12.1±2.5	8.3±2.2	1.7±1.2
	N	7	11	12	5

Table A3. Average percentage of birds eating at the PM scan samples.

		High	Low	Saline	Intact
Week 4	**% ± SEM**	2.4±1.1	5.6±1.3	6.8±1.3	5.2±1.7
	N	7	9	9	5
Week 5	**% ± SEM**	9.8±1.9	5.9±1.2	4.9±1.2	3.1±1.3
	N	7	9	9	5
Week 6	**% ± SEM**	8.5±2.3	7.0±1.4	4.7±1.1	4.8±1.4
	N	7	9	9	5
Week 7	**% ± SEM**	11.0±3.1	6.4±1.8	7.8±2.0	4.2±2.0
	N	7	12	12	5
Week 8	**% ± SEM**	9.6±2.7	5.9±1.7	3.1±1.2	4.6±2.2
	N	7	11	12	5
Week 9	**% ± SEM**	10.7±2.8	4.5±1.5	5.7±1.9	6.7±2.7
	N	7	11	12	5
Week 10	**% ± SEM**	12.5±3.1	3.2±1.3	7.3±2.0	2.1±1.5
	N	7	11	12	5
Week 11	**% ± SEM**	12.8±3.2	8.7±2.1	5.2±1.6	6.2±2.4
	N	7	11	12	5

Table A4. Average percentage of birds drinking at the AM scan samples.

		High	Low	Saline	Intact
Week 4	**% ± SEM**	7.1±1.4	14.7±4.6	7.9±1.7	4.0±1.2
	N	7	9	9	5
Week 5	**% ± SEM**	7.9±1.7	16.1±4.6	3.8±1.1	6.8±1.9
	N	7	9	9	5
Week 6	**% ± SEM**	4.3±1.3	13.6±4.7	3.4±0.9	1.5±0.9
	N	7	9	9	5
Week 7	**% ± SEM**	5.4±2.4	7.5±1.8	12.5±4.0	7.1±3.1
	N	7	12	12	5
Week 8	**% ± SEM**	7.0±2.7	4.8±1.5	4.7±1.7	3.3±1.9
	N	7	11	12	5
Week 9	**% ± SEM**	4.7±1.8	7.2±2.0	5.7±1.6	3.3±1.9
	N	7	11	12	5
Week 10	**% ± SEM**	4.4±1.8	6.4±1.8	6.3±1.7	4.2±2.0
	N	7	11	12	5
Week 11	**% ± SEM**	5.2±1.9	5.3±1.6	3.6±1.5	7.9±3.3
	N	7	11	12	5

Table A5. Average percentage of birds drinking at the PM scan samples.

		High	Low	Saline	Intact
Week 4	**% ± SEM**	3.4±1.1	12.6±4.6	7.3±1.4	6.8±2.0
	N	7	9	9	5
Week 5	**% ± SEM**	2.7±1.0	13.1±4.7	6.3±1.4	7.4±1.8
	N	7	9	9	5
Week 6	**% ± SEM**	3.3±1.1	3.9±1.1	4.8±1.2	3.3±1.4
	N	7	9	9	5
Week 7	**% ± SEM**	4.2±1.8	8.0±2.0	3.6±1.5	1.7±1.2
	N	7	12	12	5
Week 8	**% ± SEM**	4.2±1.7	1.5±0.9	2.1±1.0	2.5±1.7
	N	7	11	12	5
Week 9	**% ± SEM**	5.2±1.9	5.5±1.8	4.2±1.4	3.7±1.8
	N	7	11	12	5
Week 10	**% ± SEM**	6.3±2.1	4.5±1.5	6.8±1.9	6.7±2.6
	N	7	11	12	5
Week 11	**% ± SEM**	6.0±2.3	3.4±1.4	8.3±2.2	2.1±1.5
	N	7	11	12	5

Table A6. Average percentage of birds foraging at the AM scan samples.

		High	Low	Saline	Intact
Week 4	**% ± SEM**	17.1±3.0	17.7±2.2	15.0±2.2	20.4±3.5
	N	7	9	9	5
Week 5	**% ± SEM**	16.8±2.3	23.8±2.6	22.4±2.7	19.5±3.0
	N	7	9	9	5
Week 6	**% ± SEM**	10.7±2.0	20.9±2.2	23.4±2.5	19.5±3.1
	N	7	9	9	5
Week 7	**% ± SEM**	13.4±3.0	18.2±2.4	21.4±3.0	18.3±4.2
	N	7	12	12	5
Week 8	**% ± SEM**	15.4±3.8	30.7±3.7	22.4±3.4	23.8±5.5
	N	7	11	12	5
Week 9	**% ± SEM**	24.2±4.2	23.9±3.3	26.0±3.4	24.2±5.8
	N	7	11	12	5
Week 10	**% ± SEM**	19.5±3.6	22.5±3.4	22.9±3.0	42.1±5.8
	N	7	11	12	5
Week 11	**% ± SEM**	16.9±3.4	31.6±3.4	21.9±3.1	32.9±5.7
	N	7	11	12	5

Table A7. Average percentage of birds foraging at the PM scan samples.

		High	Low	Saline	Intact
Week 4	**% ± SEM**	11.0±2.4	15.1±2.1	14.4±2.3	11.0±2.8
	N	7	9	9	5
Week 5	**% ± SEM**	15.8±2.5	16.9±2.3	19.5±2.2	23.5±3.3
	N	7	9	9	5
Week 6	**% ± SEM**	18.6±2.8	19.4±2.1	22.2±2.5	24.0±3.5
	N	7	9	9	5
Week 7	**% ± SEM**	15.5±3.5	27.8±3.3	21.9±3.2	20.4±4.6
	N	7	12	12	5
Week 8	**% ± SEM**	18.7±3.9	28.2±3.6	19.3±3.0	25.0±4.3
	N	7	11	12	5
Week 9	**% ± SEM**	19.3±3.5	23.3±3.2	25.0±3.5	21.7±5.9
	N	7	11	12	5
Week 10	**% ± SEM**	20.1±4.0	24.1±3.2	18.2±3.0	24.2±4.9
	N	7	11	12	5
Week 11	**% ± SEM**	23.4±4.1	27.5±3.8	21.9±3.1	25.8±5.0
	N	7	11	12	5

Table A8. Average percentage of birds walking at the AM scan samples.

		High	Low	Saline	Intact
Week 4	**% ± SEM**	9.8±1.7	6.3±1.5	7.2±1.5	5.7±2.1
	N	7	9	9	5
Week 5	**% ± SEM**	11.3±2.2	9.9±1.8	23.2±3.3	10.0±2.1
	N	7	9	9	5
Week 6	**% ± SEM**	16.1±2.9	14.1±2.0	12.8±2.0	14.7±3.3
	N	7	9	9	5
Week 7	**% ± SEM**	14.9±3.5	21.4±2.9	25.0±3.2	30.0±5.7
	N	7	12	12	5
Week 8	**% ± SEM**	34.9±4.8	19.3±3.9	28.6±3.5	31.7±5.2
	N	7	11	12	5
Week 9	**% ± SEM**	19.3±3.9	25.0±3.7	29.2±3.8	37.5±5.8
	N	7	11	12	5
Week 10	**% ± SEM**	30.2±4.3	20.6±3.5	35.9±4.2	25.4±5.6
	N	7	11	12	5
Week 11	**% ± SEM**	37.5±4.6	17.2±3.0	34.4±4.1	27.1±5.3
	N	7	11	12	5

Table A9. Average percentage of birds walking at the PM scan samples.

		High	Low	Saline	Intact
Week 4	**% ± SEM**	9.2±2.3	7.4±1.5	10.0±1.8	8.0±2.0
	N	7	9	9	5
Week 5	**% ± SEM**	3.7±1.1	10.6±2.1	14.4±2.2	8.5±2.4
	N	7	9	9	5
Week 6	**% ± SEM**	11.5±2.2	18.1±3.4	10.7±1.8	15.0±3.2
	N	7	9	9	5
Week 7	**% ± SEM**	19.9±4.1	13.2±2.2	16.1±2.7	15.8±3.7
	N	7	12	12	5
Week 8	**% ± SEM**	24.5±3.8	18.0±3.7	14.6±2.7	22.9±4.5
	N	7	11	12	5
Week 9	**% ± SEM**	20.1±3.6	18.8±3.4	29.2±3.8	34.6±5.9
	N	7	11	12	5
Week 10	**% ± SEM**	27.9±4.6	17.0±3.0	28.1±4.0	21.7±5.3
	N	7	11	12	5
Week 11	**% ± SEM**	29.4±4.3	24.1±3.7	28.1±3.9	32.1±4.9
	N	7	11	12	5

Table A10. Average percentage of birds inactive at the AM scan samples.

		High	Low	Saline	Intact
Week 4	**% ± SEM**	17.2±3.5	22.0±3.2	20.5±3.2	18.3±3.9
	N	7	9	9	5
Week 5	**% ± SEM**	6.2±1.5	6.7±1.4	7.8±2.0	3.7±1.7
	N	7	9	9	5
Week 6	**% ± SEM**	7.4±2.0	5.6±1.4	8.0±1.6	4.8±2.3
	N	7	9	9	5
Week 7	**% ± SEM**	11.9±3.3	8.3±2.2	0.0±0.0	0.0±0.0
	N	7	12	12	5
Week 8	**% ± SEM**	4.2±1.7	1.1±1.1	3.1±1.2	2.1±1.5
	N	7	11	12	5
Week 9	**% ± SEM**	4.7±2.4	1.3±0.8	5.7±1.8	0.8±0.8
	N	7	11	12	5
Week 10	**% ± SEM**	0.8±0.8	2.1±1.0	3.1±1.4	1.3±1.3
	N	7	11	12	5
Week 11	**% ± SEM**	0.0±0.0	3.2±1.3	3.1±1.4	2.5±1.4
	N	7	11	12	5

Table A11. Average percentage of birds inactive at the PM scan samples.

		High	Low	Saline	Intact
Week 4	**% ± SEM**	30.7±4.4	17.7±2.7	18.2±2.9	20.2±3.2
	N	7	9	9	5
Week 5	**% ± SEM**	19.9±2.9	14.4±2.3	10.6±2.0	8.4±2.1
	N	7	9	9	5
Week 6	**% ± SEM**	7.7±1.9	5.4±1.2	6.6±1.7	10.3±2.5
	N	7	9	9	5
Week 7	**% ± SEM**	2.4±1.4	5.0±1.5	9.4±2.5	15.4±4.2
	N	7	12	12	5
Week 8	**% ± SEM**	12.5±3.7	4.5±1.9	4.7±1.8	1.3±1.3
	N	7	11	12	5
Week 9	**% ± SEM**	4.7±2.1	1.9±0.9	5.2±1.7	1.7±1.2
	N	7	11	12	5
Week 10	**% ± SEM**	2.9±1.4	4.5±1.5	4.2±1.6	3.8±2.1
	N	7	11	12	5
Week 11	**% ± SEM**	2.1±1.2	3.8±1.4	1.6±0.9	1.7±1.2
	N	7	11	12	5

Appendix III – Preliminary Ileal Data

Methods

Tissue Preparation

Ileal samples were collected from chicks at one day of age. The tissue samples were gently washed and the content flushed with phosphate buffered saline before being placed in 4% paraformaldehyde for one week. After preservation in paraformaldehyde, the tissues were dehydrated in increasing concentrations of ethanol then xylene. Following dehydration, the tissues were soaked and embedded in paraffin wax for sectioning.

Tissue Sectioning and Staining

A microtome was used to cut five micrometer sections of tissue which were then placed in warm water and collected onto slides. The slides were left on a slide warm for 30 minutes or longer to heat fix the tissues to the glass. The slides were then deparaffinized and stained with hematoxylin and eosin stains to visualize the ileal cells and nuclei.

Histological Measurements

Each stained section was viewed at 10x magnification using a light microscope connected to the computer program Motifc Images Plus 2.0. Once the intestinal section was properly focused in Motic Images Plus 2.0, the basic adjuster window was used to establish correct exposure, gain, and offset before capturing a photo of each stained intestinal section. Once a photo was captured, 3 undamaged villi were randomly selected to measure with the program's measurement tool. A line was drawn through the middle of the villi from the inner tip to the bottom of the villi near the outer layer of cells to measure villi length. A line was also drawn from the inner most part of the villi to the outer edge of the intestine to measure muscularis thickness. The eclipse tool was used to measure the perimeter of the intestine. A small undamaged portion of the perimeter was selected and the number of villi counted to extrapolate of the total villi number for the intestinal section. The data were analyzed using

an Analysis of Variance (ANOVA) in IMB SPSS Statistics 24 software (IBM Analytics, Armonk, New York). Bonferroni post-hoc tests were used to identify specific differences within the statistical model. Data were analyzed with $P \leq 0.05$ accepted as significantly different and $0.05 < P < 0.100$ accepted as a trend.

Results

Average villi length was constant between the treatments (Figure A1). High dose birds (n = 3) had an average villi length of 177.15 ± 12.53 µm, low dose birds (n = 3) had an average villi length of 172.35 ± 12.13 µm, and saline birds (n = 7) had an average villi length of 165.02 ± 14.53 µm with no significant differences between treatments (P = 0.845, F = 0.169).

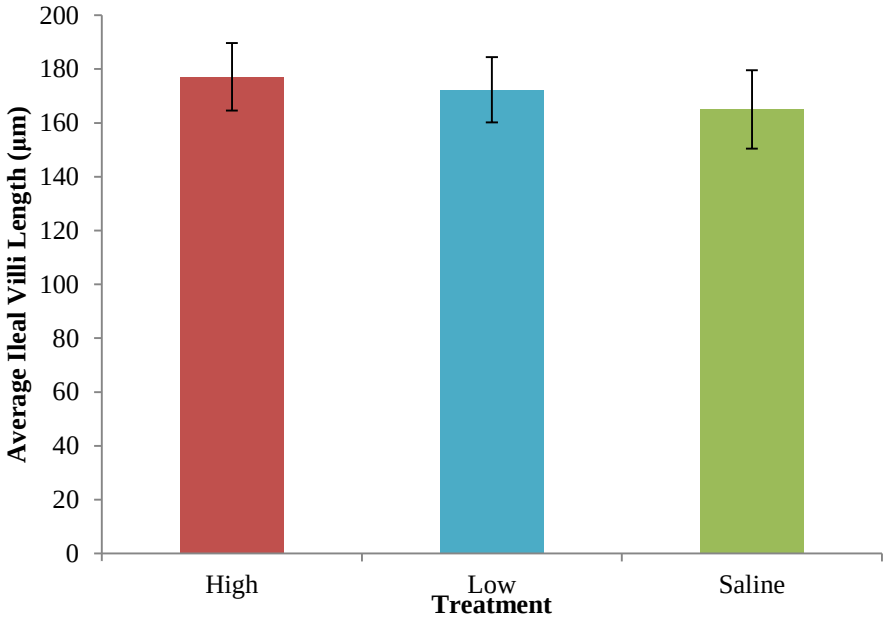

Figure A1. Average ileal villi length. There were no significant differences (P > 0.05).

Average ileal muscularis thickness was also constant between treatments (Figure A2). High dose birds (n = 3) had an average ileal muscularis thickness of 43.08 ± 2.66 µm, low dose birds (n = 3) had an average ileal muscularis thickness of 44.18 ± 2.96 µm, and saline birds (n = 7) had an average ileal muscularis thickness of 43.37 ± 2.55 µm with no significant differences between treatments (P = 0.973, F = 0.027).

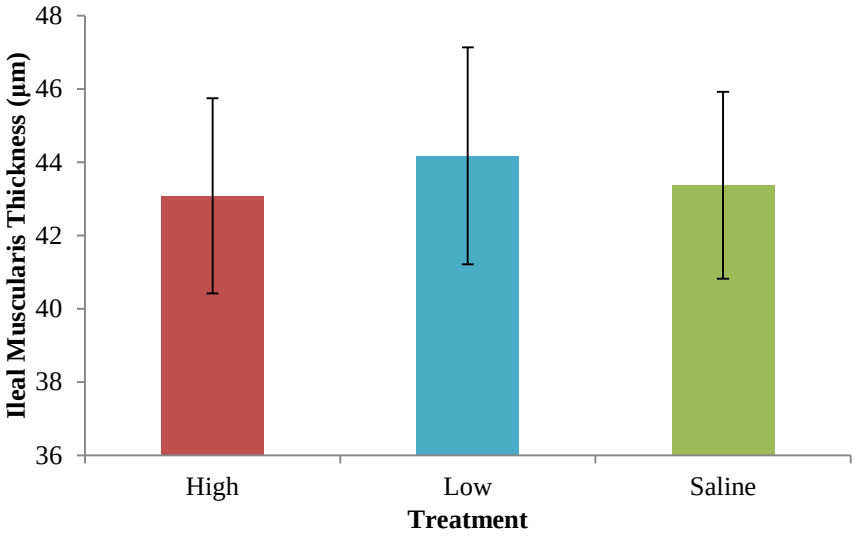

Figure A2. Average ileal muscularis thickness. There were no significant differences between treatments ($P > 0.05$).

Norepinephrine-treated birds had a slightly larger ileal circumference compared to saline birds (Figure A3). High dose birds (n = 3) had an ileal circumference of 5043.12 ± 356.83 µm, low dose birds (n = 3) had an ileal circumference of 4662.38 ± 461.92 µm, and saline birds (n = 7) had an ileal circumference of 3758.66 ± 328.31 µm with significant differences between treatments ($P = 0.049$; $F = 3.228$). High dose birds tended to have a greater ileal circumference than saline birds ($P = 0.068$).

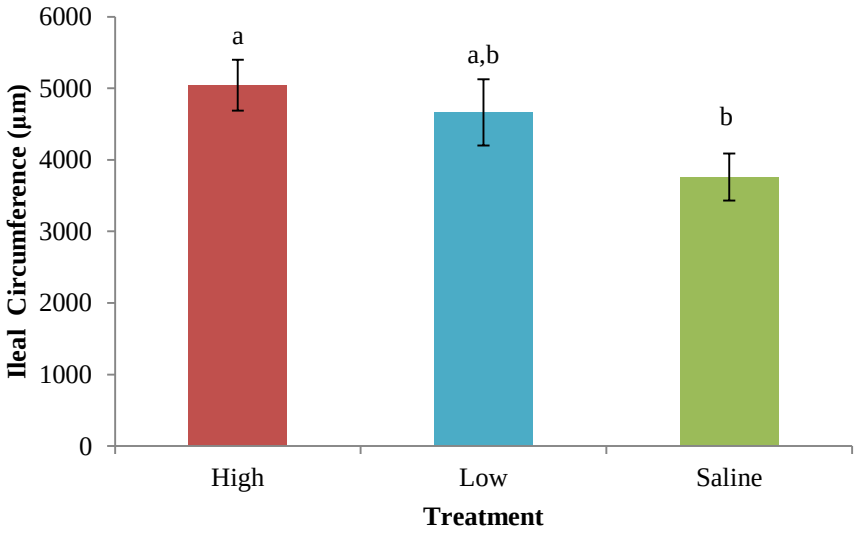

Figure A3. Average ileal circumference. Treatments that do not share a letter (abc) are significantly different. Different letters denote a significant difference between treatments ($P < 0.05$).

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