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Maternal corticosterone influences on fear responses in offspring of quail divergent stress response lines

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**MATERNAL CORTICOSTERONE INFLUENCES ON FEAR RESPONSES
IN OFFSPRING OF QUAIL DIVERGENT STRESS RESPONSE LINES**

A Thesis

Submitted to the Graduate Faculty of the
Louisiana State University and
Agricultural and Mechanical College
in partial fulfillment of the
requirements for the degree of
Master of Science

in

The Interdepartmental Program in Animal, Dairy and Poultry Sciences

by

Kyndal A. Davis
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ABSTRACT

Increased fearfulness has been associated with adrenocortical activation. Maternal corticosterone (B) treatment increases egg yolk B, and elevated B *in ovo* enhances chick avoidance of humans. Quail selected for exaggerated (high stress, HS) as opposed to reduced (low stress, LS) plasma B response to stress are more fearful, and more B is found in the egg yolks of HS than LS hens. Therefore, the underlying fearfulness (tonic immobility, TI) and timidity (hole-in-the-wall box, HWB, emergence) responses were assessed in chicks hatched from eggs of LS and HS hens implanted with silastic tubes containing no B (CON) or B (B-IMPLANT) during egg formation. In chicks 14-15 d of age, the number of inductions (INDS) required to attain TI, the latency to first alert head movement (LATHEAD), and duration of TI were determined. In chicks 21-23 d of age, the latency until first vocalization (LATVOC), numbers of vocalizations (VOCS), proportions of chicks vocalizing (PVOCS), and the latencies to head (HE) and full body (FE) emergence from a HWB were determined. LS chicks required a lower number of INDS ($P < 0.0005$) and less time to achieve LATHEAD ($P < 0.02$) than did HS ones, although stress line, maternal B-treatment, and their interaction did not affect the duration of TI. During the acclimation period of the HWB tests, more (PVOCS; $P < 0.0001$) HS chicks vocalized sooner (LATVOC; $P < 0.0001$) and more often (VOCS; $P < 0.0001$) than did LS chicks; and, while maternal implant treatment did not affect LATVOC, progeny of B-implanted hens showed a tendency towards less ($P < 0.07$) VOCS than the CONs. A line*implantation treatment interaction ($P < 0.02$) was also found for VOCS. Post-hoc analyses of the interactive VOCS means showed that the HS-CON chicks vocalized more ($P < 0.01$) than the other three similarly less vocal groups. Chicks hatched from eggs of B-IMPLANT mothers also took longer to achieve both HE ($P < 0.06$) and FE ($P < 0.05$) from the HWB than did their CON counterparts. Stress line, implantation treatment and their interaction did not alter HE or FE

responses. It was concluded that quail stress line genome may be affecting certain fear and alarm responses in chicks via the same or a different mechanism(s) that underlie(s) how maternal B increases *in ovo* B that in turn alters the fear behavior of progeny.

CHAPTER 1

INTRODUCTION

In birds, adrenocortical activation has often been associated with heightened fearfulness (Jones et al., 1988, 1992ab, 1994b, 1996, 1999; Satterlee et al., 1993; Jones and Satterlee, 1996; Cockrem, 2007). Because of the many deleterious effects of fear and distress on poultry production performance and welfare (e.g., energy wastage, feather damage, reduced growth, poor feed conversion, declines in egg production and eggshell quality, injury, pain, and higher death rates; Mills and Faure, 1990; Jones, 1996, 1997; Jones and Hocking, 1999), it is clearly important to develop ways and means to reduce stress and fearfulness. One solution may be genetic selection of commercially important poultry stocks for reduced adrenocortical responsiveness. Such selection was done early on in Japanese quail by Satterlee and Johnson (1988), who have since shown that selection for reduced (low stress, LS), as opposed to exaggerated (high stress, HS), plasma corticosterone (B) response to brief mechanical restraint is associated with many intuitively desirable traits in the LS line. These traits include a non-specific reduction in adrenal stress responsiveness to a wide variety of stressors (e.g., restraint, handling, cold, crating, feed and water deprivation, social tension, and novel objects; Jones et al., 1992b, 1994b, 2000; Jones, 1996; Cockrem et al., 2008a,b); better growth (Satterlee and Johnson, 1985); less cortical bone porosity (Satterlee and Roberts, 1990); reduced developmental instability (Satterlee et al., 2000, 2008); increased sociality (Jones et al., 2002); lower fearfulness (i.e., LS quail are less easily frightened by diverse events such as exposure to human beings, exposed areas, unfamiliar objects and places, or mechanical restraint; Jones et al., 1992a,b; 1994b, 1999; Satterlee and Jones, 1995; Jones, 1996; Jones and Satterlee, 1996; Satterlee and Marin, 2006; Kembro et al., 2008); and accelerated puberty and enhanced reproductive performance in both males (Satterlee et al., 2002, 2006, 2007; Marin and Satterlee, 2004;

Satterlee and Marin, 2004) and females (Marin et al., 2002; Satterlee and Schmidt, 2008).

Recently, B deposition into the yolks of eggs laid by genetically unremarkable (non-selected) quail hens implanted with B during egg formation has been demonstrated (Hayward et al., 2005). Hayward and Wingfield (2004) also found the same B-treatment in hens to reduce juvenile offspring growth rates and enhance stressor-induced sensitivity of the hypothalamic-pituitary-adrenal (HPA) axis in adult progeny. It should be noted that the maternal B-treatment-induced heightened HPA activity outcome reported by Hayward and Wingfield (2004) was measured by detection of plasma B response to brief capture and restraint- essentially the same genetic selection stressor used in the development of the LS and HS quail lines of Satterlee and Johnson (1988; see above).

Besides maternal B treatment, *in ovo* B-treatment *per se* also apparently has detrimental consequences on offspring hatched from B-treated eggs. For example, chicken chicks hatched from B-treated eggs show a reduced food drive (as evidenced by less willingness to cross wall barriers to obtain feed) and more fear of humans (as measured by avoidance of the experimenter) (Janczak et al., 2006). In addition, hatchlings from the eggs of B-treated yellow-legged gull hens show decreased cell-mediated immunity, a reduced rate and loudness of late embryonic vocalizations, and attenuated intensities of chick begging display (Rubolini et al., 2005). Mice offspring from mothers experiencing harsh (i.e., presumably stressful) prenatal conditions have also been shown to be less explorative of their environments (Benderlioglu et al., 2006).

Considering the above findings that associate both maternal and *in ovo* B-treatment with numerous negative consequences on production performance and increased fear behavior, and in view of the findings of Hayward et al. (2005) that both unstressed and stressed HS hens deposit more B into their yolks than do their LS counterparts, the present studies were conducted to test whether maternal B treatment would interact with known quail stress line genomic effects on

fear to further alter fear responses in the offspring of LS and HS quail. Therefore, in two separate experiments, underlying fearfulness (through tonic immobility testing; Chapter 3) and the timidity aspects of fear (through hole-in-the-wall box emergence testing; Chapter 4) was determined in juvenile offspring of control- and B-implanted LS and HS hens.

CHAPTER 2

REVIEW OF LITERATURE

2.1 Hypothalamic-Pituitary-Adrenal (HPA) Axis Control of Corticosterone Release and Its Relationship to Stress and Fear

2.1.1 The Avian Stress Hormone, Corticosterone: Stressors and Stress

Corticosterone (B) is a steroid hormone released by the adrenal glands in response to a stressful and/or fearful event. It is the major (most biologically active) adrenal glucocorticoid in avian species, often described as the avian equivalent of the perhaps more familiar mammalian glucocorticoid cortisol. When presented with a stimulus perceived as a threat (see *Stressors and Stress*, below), the avian hypothalamus is neuronally signaled to release corticotrophin-releasing hormone (CRH) into the primary capillary plexus of the hypothalamo-hypophyseal portal system (Carsia and Harvey, 2000). This CRH is carried through the portal system to the anterior lobe of the pituitary where it stimulates the release of adrenocorticotrophic hormone (ACTH). ACTH then stimulates the release of B from the adrenals into the bloodstream where B can travel back to the brain and serve as a negative feedback inhibitor of further ACTH release or travel to numerous target tissues and bring about the hormone's stress adaptation functions. Thus, B, along with epinephrine, also released from the adrenals, is primarily released to facilitate actions involved in the "fight or flight" response to a predator or other non-specific systemic stressor (Carsia and Harvey, 2000; Cockrem, 2007). Upon release, B redirects energy (carbohydrate, protein and fat metabolism) and certain behaviors towards what many consider to be basic survival tactics. For example, Boissy (1995) stated that the changes an animal's body goes through during this process cause adjustments to cardiovascular and metabolic systems that prepare the body for survival during an active response, such as "fight or flight." So, in an adaptive sense, increases in B are believed to affect functions such as foraging, as well as territorial and escape behaviors (Wingfield et. al., 1997; Wingfield and Kitaysky, 2002).

However, it is well known that when B is chronically elevated for extended periods of time, it can cause shutdowns of important and vital bodily functions (e.g., reproduction, growth, and immune responsiveness; Carsia and Harvey, 2000). Also, high levels of fear (which can lead to prolonged elevations in B) can have a number of deleterious effects on poultry such as energy wastage, feather damage, reduced growth, poor feed conversion, declines in egg production and eggshell quality, injury, pain, and higher death rates (Mills and Faure, 1990; Jones, 1996, 1997; Jones and Hocking, 1999).

Several different definitions of “stress” can be found in the scientific literature. As a result, over the years, the term stress has been grossly misunderstood and oftentimes misused. However, for the purposes of the present work, the definition of stress given in the 2007 review of Cockrem will be used. Cockrem (2007) stated that stress may perhaps best be defined as “the state of hypothalamic-pituitary-adrenal (HPA) axis activation which leads to an increase in secretion of glucocorticoids in response to the particular stressor.” Specifically, in poultry species, an increase in B secretion typically signifies when a bird is experiencing stress and the amount of B released is believed to be the best indicator of the level of stress being experienced by the animal. Cockrem (2007) has further stated that a stimulus can only be called a “stressor” if “it is considered a threat by an animal.” Only in that instance is the HPA axis activated and glucocorticoids released from the adrenal glands. If one accepts these ideas, then Cockrem suggests that stressors can be categorized in one of two ways: as physical or emotional stressors. Physical stressors produce marked changes in physical or chemical conditions of the body. Emotional stressors can bring about the same outcomes, but they require retrieval of previously stored information that can either be learned or inherited, such as that of a predatory experience.

Circulating levels of B can increase in response to any number of potentially stressful situations. There are numerous examples of stressful situations associated with management

techniques routinely used in poultry production. For example, Bedanova et al. (2007) reported levels of B become elevated when broilers are shackled for 60 and 120 seconds (prior to their processing in an abattoir). Complete deprivation of food and/or water (such as may be used to induce molt in table egg layers or to prepare broilers for processing) can cause increases in plasma B levels (Scott et al., 1983; Knowles et al., 1995) as well. Even decreases in the availability of certain nutrients within foodstuffs are known to cause elevations in blood B (e.g., in Red-legged kittiwake chicks (Kitaysky et al., 2001) and in broiler breeders (Hocking et al., 2001; de Jong et al., 2002; 2003). Elevations of B can also be caused by handling in both laying hens (Beuving and Vonder, 1978; Eskeland and Blom, 1979) and in Japanese quail (Jones et al., 2005). In addition, plasma B elevations are elicited in birds through systematic reductions in group size (Jones and Harvey, 1987), conspecific density (Nephew and Romero, 2003), capture and restraint (Jones et al., 1994b; 2000), and exposure to extreme temperatures (Edens and Siegel, 1975; Nathan et al., 1976; Beuving and Vonder, 1978). Because the stress response is non-specific in terms of stressor potency in eliciting an adrenal stress response, obviously many more examples of stressor-induced elevations in B could be offered here. But, for the sake of brevity, no more examples will be included.

2.1.2 Fear

Fear has been defined as “the state or situation in which an animal perceives a stimulus to be a threat,” and “animals considered to be in a fearful state may generate behavioral and/or physiological responses to the threat stimulus” (Cockrem, 2007). In nature, heightened fear responses may help an animal avoid a dangerous situation in order to survive and therefore pass on its genes to future generations, a logical and worthwhile strategy. Within commercial poultry production situations, however, fear responses (especially overt ones; similar to severe and

chronic stress responses) can have deleterious effects on poultry performance and animal welfare.

The concept of underlying fearfulness is discussed in detail in a series of excellent reviews by Jones (1986, 1987b, 1996, 1997). In these reviews Jones has consistently defined underlying fearfulness as “the predisposition of an animal to react easily to a potentially fearful situation” and he has generally concluded that poultry species, when in a frightened state, tend to waste energy that could otherwise support production performance, and they may injure or trample one another when exhibiting the hallmark behavior of fear- escape. Such trampling can lead to injuries, chronic pain, or even death in worst-case scenarios. For example, Mills and Faure (1990) stated that alterations in the environment of a domestic chick could trigger panic behavioral reactions, the results of which could be as lethal as suffocation (from excessive crowding) and disease.

Birds with tendencies towards fearful states can also experience higher incidences of feather loss, reduced growth, and low feed intake. For example, Craig and Swanson (1994) found that hens characterized as fearful had higher incidences of feather loss when housed individually or in groups. And, in broiler breeders, fearfulness, induced by the close proximity of a human, has been negatively correlated with feed conversion (Hemsworth et al., 1994). Hocking et al. (1997) have also associated a decrease in the feed intake of broiler breeders and layers with heightened fearfulness.

Within various poultry production schemes, high levels of fearfulness have been associated with decreased egg laying and hatchability as well. For example, Komai and Guhl (1960) studied Leghorns categorized by tameness based on human avoidance testing and found that hens having low tameness scores were poor egg layers compared to hens with high tameness scores. Decreased egg hatchability has been demonstrated by Shabalina (1984) in eggs fertilized

by broiler breeder cockerels categorized as fearful rather than calm. In addition, events such as transport from one setting to another can cause disruptions in the egg laying process (Mills and Faure, 1990) and can lead to various eggshell abnormalities (Hughes et al., 1986). Many of the detrimental effects of fear on production and welfare cited above can also lead to losses such as downgrading of broiler carcasses at slaughter and fewer eggs being sent to the hatchery from breeding flocks due to poor egg quality issues (Jones, 1997). Furthermore, poultry stocks that exhibit high levels of fearfulness can be more difficult to handle which can cause problems in daily management routines (Jones, 1997).

Generally, locomotor activity and vocalizations are also inhibited in animals in a state of fear (Jones, 1987b, 1996). However, in juvenile birds, Jones emphasizes that one needs to be careful in interpreting their locomotion and vocalizations relative to fear by considering three categorizes of fearful states that are based on levels of fear intensity. The presence of low levels of fear and novelty may provoke the subject to explore novel objects or surroundings (i.e., exhibit the so called “cautious investigation” state) and to utter “distress calls” in efforts to reinstate itself with its brood mates. Intermediate levels of fear can stimulate behaviors such as running and jumping in attempts to escape the test situation as well as high pitched peeping. In the highest fear state a bird may experience, it will almost invariably exhibit freezing behaviors (e.g., tonic immobility, TI) and vocalizations are suppressed. Ratner (1967) proposed four progressively more intense stages of fear, namely: 1) freezing, 2) fight/submission, 3) flight, and 4) immobilization. Thus, an experimenter may observe test birds exhibiting one or more of these four stages while in a fearful situation depending on the intensity of the fear they are experiencing and length of the experiment test (i.e., the behavioral “test ceiling”).

Considering the proposed progressive nature of fear states and the relationships between fear behavior exhibition, fear-eliciting stimulus intensity and length of fear test observation, it is

not surprising that a controversial literature exists on whether exacerbation or inhibition of vocalizations truly indicates fearfulness. For example, inhibitions of vocalizations have been seen in domestic fowl in response to the sound of an electronic doorbell (Phillips and Siegel, 1966). Similarly, Jones (1980) found a reduction in vocalizations from domestic chicks in response to a loud bell. Pre-test exposure to both noise and shock caused a decrease in vocalizations when group reared domestic chicks were placed in isolation (Montevecchi et al., 1973). Also, simulation of a predatory encounter (e.g. the presence of a stuffed hawk) can inhibit vocalizations in domestic chicks (Suarez and Gallup, 1981). On the other hand, Zajonc et al. (1974) have presented chicks with novel objects and found no reductions in vocalizations. Also, Kaufman and Hinde (1961) found that when chicks reared in isolation were allowed to view another chick, vocalizations were increased. Collectively, these studies suggest that the link between fear and vocalizations is not straightforward and likely involves many factors.

2.1.3 Assessments of Fear

Because poultry stocks most likely view interactions with humans and other environmental stimuli (particularly novel objects or events) as potential predatory encounters (Suarez and Gallup, 1982), and because fear has so many deleterious consequences on animal production performance and well being (see *Fear* discussion above), reduction of a bird's fear of caretakers and necessary poultry husbandry activities is imperative in poultry production. But, in order to do this, it is important to be able to, as best as possible, measure animal fearfulness. Typical behavioral tests of fear used to assess the levels or amount of fearfulness experienced by a bird include: placement of birds in a novel environment or situation (e.g., open field, emergence from a hole-in the wall box, and struggling in a crush cage tests), exposure of test animals to novel stimuli (e.g., measurement of avoidance of a novel object or experimenter), and induction of birds into TI. Although open field, avoidance of novel objects and humans, and

crush cage struggling tests were not used in the present maternal B and quail stress line studies of this thesis, their conduct is discussed in brief detail here to educate the reader about these important behavioral tests of fear, tests that were used to previously detect fear response differences between birds of the LS and HS quail lines (see *Fear and Corticosterone* section, below).

- **Open Field**

Fearfulness can be determined with the use of “open field” testing. It is important to note here that the use of the term open field is somewhat of a misnomer as open field test apparatuses do not have limitless boundaries, rather open fields are typically simulated by construction and use of test ‘boxes’ that have both defined lengths and widths. The apparatus used in open field testing also varies from study to study but, clearly, all open fields should be considered to be quite novel to the test bird. For example, the walls and floors of most open field test boxes are commonly painted white or matte-white which is thought to greatly intensify the test stimulus’ novelty since visual clues are significantly dampened under such situations.

Open field testing is done by capturing a bird from its familiar (e.g., home cage or pen) environment and then transporting it, and placing it inside of the novel (frightening) open field. The experimenter then observes the behavior of the animal during the time it spends inside the open field apparatus using a fixed “test ceiling” time (typically 5 – 10 min). Some of the commonly observed behaviors that are recorded during open field tests include the time spent: feeding, conducting organized exploration of the environment (e.g., pecking and walking), peeping (vocalizations), and freezing (e.g., standing still, sitting, lying, and eye closure) (Jones, 1987b). Open field behavioral outcomes are generally interpreted along the lines of the hypothesis that greater fear is associated with “silence and inactivity” as discussed above. Again, it is important to note that Suarez and Gallup (1981) performed a series of experiments that led

to the conclusion that open field testing contains significant predatory overtones (e.g., bird capture and transport to the test apparatus). Clearly open field testing has other inherent fear-inducting conditions that may or may not be construed by the test subject as predatory as well—for example, the novelty of the open field *per se*, isolation from familiar home environment conspecifics, loss of conspecific sight, olfactory, and auditory stimuli, etc.). It is important to note here that, in comparison to LS quail, HS quail show many different heightened fear behaviors in open field testing (Jones et al., 1992a, 1994b; Satterlee and Marin, 2006; Kembro et al., 2008; see *Quail Stress Response Lines*, p. 19-21).

- **Avoidance of Novel Objects and Humans**

The novel object test usually consists of the presentation of a novel object into an area to which an animal has been allowed an “acclimation period”. A novel object can also be introduced into an animal’s home pen. During this test, the animal’s avoidance of a novel object is measured through their proximity to (including ambulation towards), number of contacts with, and/or duration of contacts with the novel object, as well as other avoidance-related behaviors. A novel object can be anything previously unknown (i.e., something “foreign”) to the test subject. To increase a novel object’s “fear value,” oftentimes the stimulus object chosen is typically brightly- and/or multi-colored. For example, the use of wooden rods covered with colored strips of plastic tape placed within the cage or in the food trough has been used to elicit fear responses in caged chickens (Jones, 1985). Other objects that have been successfully used to elicit fear behavior in novel object tests include: pencils, metronomes, fishing floats, Christmas ornaments, and cones (Jones 1987b; Jones, 1996; Cockrem et al. 2008b). It is tempting to speculate that the outcomes of novel object testing may have been what has led to the concept of “environmental enrichment” as an animal welfare tool. For example, it has been shown that

enriched environments can reduce a domestic chick's avoidance of novel objects (Jones and Waddington, 1992).

Suarez and Gallup (1982) demonstrated that poultry view human beings as predators. Thus, it is no surprise that levels of fear in poultry are also measured through tests of avoidance of the experimenter. Such testing can be carried out in various ways. One method involves an experimenter sitting in a chair in the center of a floor pen containing chicks. The proximity of the chicks to the human is then determined usually by numbering imaginary zones around the occupied chair. The chicks are then given an "avoidance score" of 1 - 5 (either in ascending or descending proximity to the experimenter) based on a total of their positions over a certain time frame. Another method is called the "box plus experimenter" method. This test uses the same premise as the above method; however, during the box plus experimenter test, the human is seated behind a wire mesh wall at the end of an arena. The chick is scored on its approach or avoidance to the experimenter behaviors. Again, higher avoidance suggests higher fear levels. Yet another human avoidance test is called the "approaching human" test (Jones, 1996). This test is used primarily in commercial poultry situations. It uses a video camera strapped onto the experimenter's shoulder which tapes the reactions of the birds housed on the floor as the experimenter walks among them pausing intermittently at times to record the animals' behavior. The videotapes are analyzed following the testing by replay of the videos and counting the numbers of birds in close proximity to the experimenter. More birds within a visual field (i.e., in close proximity to the experimenter) is considered to be indicative of less fear. It is again worthy to note here that increased human-animal contact (e. g., regular handling; Jones and Faure, 1981; Jones and Waddington, 1993) as well as environmental enrichment (Jones and Waddington, 1992) can decrease human avoidance responses (and therefore fearfulness of humans) in domestic chicks. The reader is also again reminded here that, in comparison to LS quail, HS

quail show many different heightened fear behaviors in both avoidance of novel objects and avoidance of human tests (Jones et al., 1994b; Satterlee et al., 1999, unpublished data; Cockrem et al., 2008b; see *Quail Stress Response Lines*, p. 19-21).

- **Struggling in a Crush Cage**

The use of struggling in a crush cage as a fear testing apparatus has also been likened to fear of predator responses exhibited by poultry. Such testing is similar to handling in that the bird is restrained in an apparatus (crush cage) and is unable to escape. When placed in a crush cage situation, typically, a movable cage wall is pushed up flush against the bird and fixed in place. This allows for respiration but prevents most gross movements although head and leg movements can still be made. Common fear behaviors recorded in crush cage testing include: observation of the latencies to first vocalize and struggle, the numbers of vocalizations and struggling episodes, and the total time spent struggling during testing. As stated earlier, fear is generally thought to have an inhibitory effect on vocalizations and activity- the so-called “silence and inactivity” hypotheses (Jones, 1987b, 1996). Thus, birds that have longer latencies to first vocalize and struggle, fewer numbers of vocalizations and struggling bouts, and less total time spent struggling during testing in a crush cage are generally viewed as being more fearful. Important to the present thesis studies, Jones et al. (2000) found restraint in a metal crush cage for 5 min to be associated with shorter latencies to vocalize and struggle in LS than in HS quail. In addition, LS quail showed greater numbers of struggling bouts and a higher total time spent struggling than did their HS counterparts. It is further worth noting here that, in crush cage testing, quail selected for short durations of tonic immobility (i.e., presumably less fearful birds; see *Tonic Immobility* section below) struggled more often than those selected for long tonic immobility reactions (Jones et al., 1994a).

- **Tonic Immobility (TI)**

Jones (1986) has defined tonic immobility (TI) as “an unlearned fear-potentiated, catatonic-like state of reduced responsiveness, induced by brief manual restraint.” Birds that exhibit a high susceptibility to TI and longer duration of TI once successfully induced into TI are believed to be more likely to show high fear reactions in other potentially fearful situations. TI is thought to be an anti-predatory reaction which occurs in a number of species (Ratner, 1967) but is a very pronounced reaction in both rabbits (Ewell et al., 1981) and avian species (Gallup, 1977). Theoretically, the purpose of the immobility state of TI is to lessen a predator’s interest in the prey by the prey’s decrease in struggling (Gallup, 1977; Jones, 1986, 1987; Boissy, 1995; Korte, 2001). Thus, the underlying behavioral themes of the TI reaction state are similar to those of struggling in a crush cage in that both tests measure logical extensions of behaviors (i.e., decreased struggling and eventual immobility) that exemplify anti-predatory reactivity.

TI is generally induced in an experimental setting by holding a bird inverted on its back for approximately 15 s. A successful induction into TI is one in which the bird remains on its back after the experimenter’s hands are released. Jones (1986) has hypothesized that chicks may experience TI in three stages or levels. The first level involves sharp vocalizations and open eyes. The second consists of eye fluttering and a decrease in vocalizations. During the third and deepest stage of TI the bird is quiet, its eyes are fully closed, and it exhibits body twitching and head bobbing. The third state of TI can last for a few seconds or for many hours.

Durations of TI can be affected by various factors; and, certain factors linked to fear responses (especially treatments that involve adrenocortical stress responses) can apparently cause increases in TI duration and thus, underlying fearfulness. For example, Jones et al. (1988) found that when chicken hens were administered physiological levels of B via mini-osmotic-pump implants, they had significantly longer durations of TI in comparison to control hens. Also, in chickens, exposure to adrenaline (epinephrine) prior to testing can increase the durations

of TI (Braud and Ginsburg, 1973). Zulkifli et al. (2000) have also shown that broiler birds subjected to either inverted handling (IH) as opposed to upright handling (UH) had longer durations of TI. Presumably, IH would be more stressful to birds than UH. In domestic chickens, fowl that are purportedly a more “flighty” breed, i.e., White Leghorns, exhibit longer durations of TI than do ISA Brown strain chickens that are thought to be more “docile” (Jones, 1987a). Suarez and Gallup (1981) reported that chickens given an open field test (undoubtedly a stressful situation) prior to TI testing have longer durations of TI and, similarly, Gallup et al. (1970a, b) found that, in chickens, pre-TI testing exposure to mild electrical shock and loud noise increased the durations of TI as well. Interestingly, just visual contact with or sight of an experimenter or caretaker can influence the length of TI. For example, Gallup et al. (1972) have shown that chicks experience longer durations of TI when they have clear views of an experimenter’s eyes and Jones (1985) found hens housed on the top tier of a battery deck (i.e., hens that would have greater daily human-animal interactions and therefore likely more eye-to-eye contacts with humans) to have longer durations of TI compared to those housed on the middle tier. Gallup (1977) stated “perhaps the significance of eye contact is that it allows the prey to gauge the attention of the predator, and thereby provides information about potential opportunities for escape when the predator becomes distracted or disinterested.” Considering these words of Gallup (1977) and the findings from Suarez and Gallup (1982) that humans are viewed as predators by poultry it is not surprising that care is always taken by experimenters during TI testing to avoid eye contact with the bird being tested. Clearly, eye contact between and experiment and the test bird prolongs the duration of TI.

Methods of reducing the TI response have been proposed. For example, Jones (1992) showed that in chicks (*Gallus gallus domesticus*), positive human contact and even observance of another bird receiving positive contact with a human can shorten TI durations. Jones et al.

(1996) also found that quail treated with a vitamin C solution in their drinking water showed shorter TI reactions than a control (untreated water) group. Because vitamin C interferes with the synthesis of B at several steps in the pathway of adrenal corticosteroidogenesis, it has been proposed that vitamin C-induced fear reduction may be the result of a reduction in levels of blood B. Gallup et al. (1971a) have also found that giving a tranquilizer specifically developed for domestic fowl can reduce the intensity TI reactions. But, perhaps the best (least expensive, simpler, more practical, permanent and welfare-friendly) solution for reducing TI responses (i.e., underlying fearfulness) in poultry may be to genetically select for reduced adrenocortical responsiveness to stress. This was clearly pointed out in an extensive review by Jones (1996) that discussed the many fear reduction outcomes (including reduction in TI) known to exist in the LS quail line originally selected by Satterlee and Johnson (1988) for reduced plasma B response to brief manual restraint (see *Quail Stress Response Lines* section below).

The age of the bird can also play a role in affecting TI responses seen during TI testing (Jones, 1986). For example, immature birds are less experienced and elicit more genuine reactions to fear testing since, due to their young age, their numbers of experiences with stressors are lower. As birds age, however, fear reactions likely become dulled as older birds become less reactive to certain stimuli they perceived to be more frightening when they were younger. Indeed, birds should not be tested too soon post-hatch since there is apparently a lack of TI reaction in very young hatchlings. For example, the TI response has been reported to occur no earlier than 7-10 d of age in poultry (Ratner and Thompson, 1960; Salzen, 1963). For this reason, in the present studies, chicks of 13 - 23 d of age were used to assess underlying fearfulness via TI testing (Chapter 3) and the timidity aspects of fear using emergence tests (Chapter 4).

- **Emergence**

Levels of timidity (i.e., fear of unknown or unfamiliar areas) are usually determined using some sort of emergence testing that measures the time it takes an animal to move from a more familiar environment to an unfamiliar one. Emergence testing is most commonly used in rodents, but is becoming increasingly more studied in avian species, especially domestic ones.

In “hole-in-the-wall box” (HWB) testing, it is assumed that animals with longer latencies to emerge from a dark, and therefore presumably “safer” compartment, into a lighted space (compartment) are exhibiting more fear than those with shorter latencies to emerge (Jones, 1987b, 1996). The HWB test was adapted for use in domestic fowl by Jones (1979). When examining the effects of early enrichment on timidity in domestic chicks, Jones (1982, 1992) found that enriched chicks had shorter emergence latencies from the HWB than those chicks that were not enriched. When studying the HWB responses in birds from strains deemed “active” or “inactive,” Jones et al. (1982) found that birds from the active strain had higher emergence latencies. Jones and Mills (1983) also found when studying birds of a “flighty” vs. “docile” strain that those from the flighty (presumably the more fearful) strain had longer latencies to emergence than what was found in the docile birds. Of most importance to the presently proposed studies, the reader is once again reminded here that LS quail have been shown to emerge from a HWB sooner than do HS chicks (Jones et al., 1999; Satterlee and Jones, 1995; and, see *Quail Stress Response Lines* section below for further detail).

2.1.4 Relationship Between Fear and Corticosterone

Clearly, increases in blood levels of B occur when a bird is presented with a potent enough fearful situation. For example, presentation with a novel object can elicit an increase in B levels in chickens and in Japanese quail (Murphy, 1977; Richard et al., 2007). As stated previously, humans are viewed by poultry as predators, therefore human contact typically triggers B release. However, the frequency of bird contact with humans can alter the levels of

fear experienced by the bird. For example, Hemsworth et al. (1994) measured the level of plasma B in birds receiving either regular human contact or minimal human contact and found that birds with minimal human contact had higher levels of plasma B in response to 12 min of handling than those who received human handling regularly. Several other studies as well have found similar results of reduced B release in response to human contact by increasing the amount of positive interactions between an experimenter and the birds (Jones and Faure, 1981; Jones and Waddington, 1992, 1993). This relationship is not always straightforward, however, as demonstrated by Turkeyilmaz and Fidan (2006) who found that when broiler chicks were exposed to human contact no effects on B levels were evident.

2.1.5 Quail Stress Response Lines

Jones (1996) has proposed that genetic selection for reduced adrenocortical responsiveness to stress may “be the quickest and most reliable method of promoting desirable, ‘welfare-friendly’ characteristics across whole populations.” With such an idea in mind, early on, Satterlee and Johnson (1988) genetically selected two Japanese quail lines for divergent stress responsiveness. Many studies of these stress response lines over the last 20 years have shown that selection for reduced (low stress, LS), as opposed to exaggerated (high stress, HS), plasma B response to brief mechanical restraint is associated with many intuitively desirable traits, both physiological and behavioral, in the LS line. These traits include: a non-specific reduction in adrenal stress responsiveness to a wide variety of other stressors in addition to the genetic selection stressor of manual restraint (e.g., handling, cold, crating, feed and water deprivation, and social tension; Jones et al., 1992b, 1994b, 2000; Jones, 1996; Cockrem et al., 2007); improved growth (Satterlee and Johnson, 1985); less cortical bone porosity (Satterlee and Roberts, 1990); reduced developmental instability (Satterlee et al., 2000, 2008); reduced fear (Jones et al., 1988, 1992a, b, 1994b, 1996, 1999; Satterlee et al., 1993; Jones and Satterlee, 1996;

Kembro et al., 2008); increased sociality (Jones et al., 2002); and, accelerated puberty and enhanced reproductive performance in both males (Satterlee et al., 2002, 2006, 2007; Marin and Satterlee, 2004; Satterlee and Marin, 2004) and females (Marin et al., 2002; Satterlee and Schmidt, 2008).

For the sake of brevity and because the present thesis studies deal specifically with only fear response differences in the LS and HS quail stress lines, only the reduction in fear traits that have accompanied selection of the LS line mentioned above will be reviewed in more detail here. LS quail are known to both freeze less and ambulate more in open field tests than do their HS counterparts (Jones et al., 1992a; Satterlee and Marin, 2006). Kembro et al. (2008) have also performed detrended fluctuation analyses of open field locomotion behavior in LS and HS quail and found that LS quail walk sooner, more often, and have a more complex ambulatory pattern in comparison with HS quail.

Differences in various TI fear reactions in the LS and HS quail lines have been documented as well. Specifically, in response to treatment with a short-latency stressor (Satterlee et al., 1993) or following overnight cooping (Jones et al., 1992b), LS quail are more resistant to induction into TI than HS quail and HS quail are known to have longer durations of TI than LS quail under the latter scenario (Jones et al., 1992b). Also, Jones and Satterlee (1996) found that LS quail show less exaggerated B responses to manual restraint when confined in a crush cage and they struggle sooner and more often than do HS quail. The reader is reminded here of the parallels between struggling behavior in both TI and crush cage tests as such behaviors relate to proposed predatory encounters.

Studies by Jones et al. (1999) and Satterlee and Jones (1995) found that LS quail have shorter latencies to head and full body emergence from a HWB than do HS quail. Jones et al. (1999) also found that LS quail vocalized sooner than HS quail while in the dark compartment of

the HWB. Furthermore, Cockrem et al., (2008b) found that, when exposed to a novel object (peppermint striped Christmas ball ornament hung in their cages), LS quail tended to exhibit less fearful reactions to such novelty (e.g., passes by the ball passes and pecks at it) than did HS quail. Similarly, LS quail have shown less avoidance of a multi- and brightly-colored fishing float placed in their feed troughs than HS quail (Satterlee and Jones, 1999, unpublished data). Moreover, the heightened fear of novelty in HS quail is apparently extended to include human beings. For example, Jones et al. (1994b) found that, in response to a nearby human, LS quail exhibit less fear and avoidance behavior (i.e., crouching and attempting to escape behaviors) than do HS quail.

Collectively, if one interprets the outcomes of all the quail stress line studies just cited using the hypothesis that fear is consistent with “silence and inactivity,” then it can be concluded that the LS quail have been shown overwhelmingly to be less fearful than their HS counterparts in a variety of fear assessment test situations (open field, TI, avoidance of novel objects and humans, emergence testing, and struggling in a crush cage).

2.2 Maternal Corticosterone Effects on Offspring Production Performance, Fear, and Other Behaviors

In unstressed avian species, low levels of maternal B are naturally deposited into egg yolks during egg formation. However, more hen deposition of *in ovo* B appears to occur during stressful events (Sanio et al., 2005) and certainly when mothers or eggs *per se* are purposely treated with B (Eriksen et al., 2003; Hayward and Wingfield, 2004). Of particular importance here is that when *in ovo* levels of B are heightened, developing embryos can be exposed to higher than normal levels of B during embryogenesis that, in turn, can apparently dramatically alter the physiology and behavior of both hatchlings and adult offspring.

Hayward and Wingfield (2004) were the first and so far only researchers to challenge reproductively active Japanese quail hens with B (via subcutaneous silastic B-filled implants)

and simultaneously measure the concentrations of B in both the hens and their egg yolks. They found hens with B implants had higher levels of B that appeared to be associated with elevated B in their egg yolks than hens given sham implants. Furthermore, offspring from the hens fitted with B implants had slower growth rates during the first 7 d of life and heightened activity of their HPA axes in response to brief restraint as adults. Saino et al. (2005) injected the eggs of barn swallows with B to compare their hatchability and the performance of the juvenile hatchling progeny with that of two control groups of eggs (that were either sham inoculated or left untreated). Eggs injected with B showed diminished hatchability compared to both control groups, and the hatchlings of B-treated eggs showed reduced body weight and slower plumage development. Eriksen et al. (2003) have also reported similar results of reduced growth in chicks hatched from chicken eggs injected with B. These workers further found that chicks hatched from B-treated eggs had higher fluctuating asymmetry in their tarsus bone lengths as adults. It should also be noted here that more developmental instability (i.e., fluctuating asymmetry) has been reported in HS than in LS hens in their respective metatarsus (Satterlee et al., 2000) and tibiotarsus (Satterlee et al., 2008) bone and face (Satterlee et al., 2000, 2008) lengths.

Other reports in avian species that have shown maternal stress, maternal B treatment, or *in ovo* B-treatment can affect the behavior of chicks hatched from eggs of such treatments include the studies of Rubolini et al. (2005) and Janczak et al. (2006, 2007a). Specifically, Rubolini et al. (2005) found eggs of yellow-legged gull hens treated with B produce chicks that show decreased cell-mediated immunity, a reduced rate and loudness of late embryonic vocalizations, and attenuated intensities of chick begging display. Janczak et al. (2006) found chicks hatched from B-treated eggs show: a reduced food drive (as evidenced by less willingness to cross wall barriers to obtain feed) and more fear of humans (as measured by avoidance); and,

when hens were exposed to a stress treatment that increased the amount of B deposited into their egg yolks, Janczak et al. (2007a) found the offspring of these hens exhibited longer durations of TI and they ate less than the controls.

In viviparous animals (e.g., mammals), when a mother experiences elevations in her circulating glucocorticoids during pregnancy, her gestating embryos can also be exposed to these hormonal changes. This effect is due to the ever-present placental connection between the mother and her developing fetuses and the consequences of such exposures on her offspring. Although the maternal hormonal delivery system is different from the oviparous (egg laying, non-placental) birds, many maternal stress hormone effects on mammalian offspring are, nevertheless, strikingly similar to those that have been found so far in avians. For example, the offspring of prenatally stressed rats release more B (Henry et al., 1994) and exhibit more escape behavior (Vallee et al., 1997) in response to novelty. Benderlioglu et al. (2006) have also found that rat offspring of prenatally stressed mothers exhibit more freezing behaviors and less exploration of new environments than progeny of untreated mothers. In juvenile rhesus monkeys, prenatal stress can cause abnormal social behaviors such as mutual clinging (Clarke and Schneider, 1993). And, in gilts, Otten et al. (2007) found repeated injections of ACTH during late-gestation induces the release of the mammalian stress hormone cortisol that was associated with more escape behavior during open field testing of the piglets that were derived from litters of ACTH-than control-treated mothers.

Several biological reasons have been offered to explain why stress-induced maternal transfer of larger portions of her circulating glucocorticoid pool to offspring occurs. Groothuis et al. (2005) suggests that, in certain instances, embryonic exposure to higher levels of glucocorticoids may have positive consequences on neonates, and maternal hormone transmission may be a path by which the mother hormonally “communicates” with the offspring

post-birth. An example of such thinking is the occurrence in rodents and lizards of higher anxiety (Valleé et al., 1997; Lordi et al. 2001) in the offspring of prenatally stressed mothers that purportedly allow such progeny to better avoid risks and therefore survive environments perceived to be harsh by mothers. By producing offspring that are more cautious, the mother thereby ensures their survival in an environment perceived as being less safe. Another example would be the purposeful production of lizard offspring with slower growth rates associated with exposure to prenatal stress (Meylan and Clobert, 2005) as a survival tactic that occurs supposedly when a mother is in an environment with poor food availability- an adaptation for the next generation of an ability to endure the situation of scarcity of food. A high number of predators in the environment have also been proposed as stimuli that may result in increased maternal B release during gestation (Groothuis et al., 2005). It is possible, at least in wild birds, that mothers can even somehow determine whether a novel animal is a threat and adjust their parental *in ovo* deposition of B accordingly. For example, when female barn swallows are treated with exposure to either a predator or a herbivorous animal, females exposed to the predator lay eggs with greater B concentrations than those hens exposed to the herbivore (Saino et al., 2005).

Non-human animal research in the area of maternal B effects on progeny are also important because there is considerable support for the idea that findings of such animal prenatal stress studies may be applicable to humans. Indeed, in a recent review by Austin et al. (2005) entitled, "Prenatal stress, the hypothalamic-pituitary axis and offspring neurobehavior," the authors made just such a connection. Furthermore, Lay and Wilson (2002) have proposed that poultry studies, due to the oviparous nature of birds that better allows control of levels of supplemental B, may afford researchers with one of the best animal models to study prenatal stress and its effects on offspring. To test this contention these workers performed an experiment

using developing chicken embryos that were treated with either B, elevated incubator temperature (HEAT), or no B (controls) on day 16 of egg incubation. Chicks hatched from HEAT-treated eggs had lower body weights than chicks derived from the other treatments, and the chicks from such thermally treated eggs remained lighter than all other chicks throughout the study. Chicks from B-treated eggs tended to have higher levels of plasma B than chicks from the HEAT and control egg treatments. At 16 wk of age, cocks from control eggs were more aggressive than cocks from HEAT and B-treatments. Cocks hatched from B-treated eggs were also chased more often than the cocks from the other treatment groups. Based on these results the authors concluded that although only certain effects of prenatal stress were found in offspring hatched from both B- and HEAT-treated eggs, with further research, the system may be refined as an appropriate (optimized) model to study the effects of prenatal stress on offspring phenotype.

2.3 Rationale for the Present Study

As discussed in detail above, when compared to LS quail, HS quail clearly show an exaggerated plasma B response to many different non-specific systemic stressors as well as heightened fearfulness in multiple tests of fear. Furthermore, the effects of maternal B in genetically unremarkable (non-selected) birds in dampening the HPA axis and fear responsiveness of their offspring have also been reviewed. Because Hayward et al. (2005) found genetically unremarkable quail hens supplemented with B deposit significantly more B in the yolks of their eggs and produce adult offspring with an exaggerated HPA responsiveness to brief restraint (a trait shared with the HS line), and because Janczak et al. (2006) were able to associate *in ovo* B-treatment with greater fear in hatchling chicks, and because Hayward et al. (2005) also found HS hens deposit more B into their egg yolks than do LS hens, it was hypothesized that maternal B treatment would interact with the divergent LS and HS quail stress

genomes to further (beyond known genomic effects) alter fear responses in the offspring of the two quail stress lines. The present studies tested this hypothesis. Specifically, during egg formation, LS and HS mothers were given silastic implants filled with either B or no-B (controls) and then their juvenile offspring were tested for differences in underlying fearfulness (via tonic immobility tests; Chapter 3) and in the timidity aspects of fear (using hole-in-the-wall box emergence testing; Chapter 4).

CHAPTER 3

TONIC IMMOBILITY RESPONSES IN OFFSPRING OF JAPANESE QUAIL STRESS LINE HENS TREATED WITH CORTICOSTERONE DURING EGG FORMATION

3.1 Introduction

Tonic immobility (TI) has long been considered the “gold standard” for measuring fearfulness in animals (Gallup, 1977, 1979; Jones, 1987b; Jones 1996). The TI reaction occurs in response to a frightening event, or in nature, a predatory encounter (Gallup et al., 1971b; Gallup, 1977). Theoretically, the longer a bird remains in tonic immobility the higher its level of fearfulness (Jones, 1987b). There is also a voluminous literature that supports the contention that adrenocortical activation is associated with heightened fearfulness (Jones et al., 1988, 1992ab, 1994b, 1996, 1999; Satterlee et al., 1993; Jones and Satterlee, 1996; Cockrem, 2007).

Japanese quail from lines genetically selected for either reduced (low stress, LS) or exaggerated (high stress, HS) adrenocortical response to brief immobilization (Satterlee and Johnson, 1988) have been examined for differences in their TI responses. In two separate studies quail of the LS line were shown to require more attempts to successfully induce them into TI, and LS quail exhibited shorter durations of TI and latencies to their first head movement (Jones et al., 1992; Satterlee et al, 1993).

Non-selected quail hens implanted with B during egg formation have increased levels of plasma B that is associated with greater deposition of B into their egg yolks (Hayward and Wingfield, 2004). Such treatment dampens early growth rates of chicks and enhances stressor-induced sensitivity of the HPA axis in adult progeny of B-treated hens. Chicks hatched from *in ovo* B treatments also show a reduced food drive and more fear of humans (Janczak et al., 2006). Hayward et al. (2005) also found both unstressed and stressed HS hens to deposit more B into their egg yolks than do LS hens. Therefore, the present study was conducted to determine

whether maternal B treatment would interact with the divergent LS and HS quail stress genomes to alter the underlying fearfulness of juvenile offspring of the two quail stress lines.

3.2 Materials and Methods

3.2.1 Genetic Stocks and Animal Husbandry

Offspring from generation (G)₃₈ of two lines selected for either low (low stress, LS) or high (high stress, HS) plasma B response to brief immobilization were studied. Satterlee and Johnson (1988) have described the genetics that underlie the first 12 generations of pedigree selection, and the most recent genetic history of the lines, up to G₃₄, is discussed in detail elsewhere (Satterlee et al., 2000; Marin and Satterlee, 2004; Satterlee et al., 2006). Although line differences in levels of plasma B were not measured in the present study, recent findings in the stress lines attest to the maintenance of divergent adrenocortical responsiveness to a variety of non-specific systemic stressors. Indeed, Satterlee et al. (2007) have most recently offered explanations as to why the gene(s) that control the adrenocortical responsiveness trait in these lines have likely become fixed.

At 29 wk of age, ninety-six hens (48 LS + 48 HS) were pair housed with a non-sibling, same-line male in a single cage of one of two Alternative Cage Designs (Alternative Design Manufacturing and Supply, Inc., Siloam Springs, AR) four-tier cage batteries. Each battery contained 48 pedigree-style breeder cages (individual cage dimensions were 50.8 x 15.2 x 26.7 cm, length x width x height, respectively). Care was taken to insure that each of the breeding pairs selected, while randomly selected from larger family populations within each line of the same hatch, constituted, as nearly as possible, equal representation of the 12 different families that make up each line. A breeder ration (21 % CP; 2,750 kcal ME/kg) and water was provided to the birds *ad libitum*. The daily photostimulatory cycle was 14 L: 10 D (approximately 280 lux

during the lighted portion of the day); lights-on was at 06:00 h and lights-off was at 20:00 h daily. Daily maintenance and feeding chores were conducted at 08:00 h daily.

3.2.2 Hen Treatments

At 33 wk of age, half of the hens from each line ($n = 24$ birds/line) were individually fitted with 16 mm silastic-tube (Dow Corning, Midland, MI; Cat. No. 508-006) implants containing either corticosterone (B; Sigma–Aldrich Co., Atlanta, GA; Cat. No. C2505) or no B (controls, CON). Thus, four treatment combinations resulted: LS-controls, LS-B-implants, HS-controls, and HS-B-implants. Implants were placed s.c. in the back of the neck using a No. 10 biopsy needle (Becton Dickinson, Franklin Lakes, NJ). The implant tubes were sealed at one end with silicone sealant and were left open on the other end. Hens were allowed a 10 d acclimation period to allow sufficient time for maternal B deposition into the eggs of B-treated hens (Hayward and Wingfield, 2004) in response to their implantation treatments. Eggs were then collected daily, identified by pencil markings as to their origin by hen line and implantation treatment, and stored at 18 C until incubation. Egg collection lasted for 3 wk, and these eggs were then set together into an incubator (NatureForm NMC 2000; NatureForm Hatchery Systems, Jacksonville, FL). During the first 14 d of incubation, eggs were turned 6 times a day and subjected to 37.5° C and 62 % RH. Upon transfer of the eggs to a second NMC 2000 hatcher unit on Day 14, eggs were no longer turned and incubation conditions were changed to 37.2° C and 69 % RH.

3.2.3 Offspring and Variables Measured

At hatch, chicks were leg banded with appropriate different color and uniquely numbered leg bands that allowed their identification with the four line*implantation treatments (LS-CON, LS-B-implant, HS-CON, and HS-B-implant). Chicks were brooded, all treatments equally co-mingled, in three confinement rings (approximately 260 chicks/ring). This arrangement resulted

in about 65 chicks from each treatment combination being represented in each ring. The brooding ring areas were of identical construction- each ring was 1.2 m in diameter, heated with two 125-watt incandescent lamps, and had pine wood shavings as a floor substrate. Chicks were fed a quail starter ration (28% CP; 2800 Kcal ME/kg) and given water *ad libitum*. Brooding temperatures and their change with time were similar to those used by Jones and Satterlee (1996).

At 14 d of age, 80 chicks (20 from each of the four stress line*implantation treatment combinations) were selected for TI studies. Individuals were randomly captured throughout the test day in equal rotation from each of the three confinement rings until the above sample numbers were achieved. Upon capture, a test chick was removed to a separate room (i.e., the TI test apparatus was located in a quiet area, approximately 13 m away from the live-bird facility, and free from bird noises and human traffic) and its TI responses were measured as follows. Placement of a bird on its back in a 120° V-shaped polystyrene cradle covered by a white cloth was used to induce TI. Chicks were restrained in this dorsal recumbent position for 15 s using one of the experimenter's hands placed on the sternum and the other lightly cupping the head. Successful induction into TI was defined as when a chick attained TI lasting for at least 10 s. If a chick did not achieve TI on the first try, additional induction attempts were made. If induction into TI was not accomplished after five attempts, a test subject was deemed unsusceptible to TI and given a score of "5" for the number of induction attempts (INDS) needed to induce TI. Following a successful induction into TI, the experimenter quietly retreated to a non-intrusive position (approximately 2 m away from the TI cradle) while remaining in full sight of the chick. The experimenter then observed and recorded: the latency from the end of induction into TI until the first alert head movement (generally a gross, scanning behavior; LATHEAD, s) and the duration of TI (the length of time between the end of induction to observation of a chick self

righting response) (TI, s). Maximum scores of 600 s (using a test ceiling of 10 min) were assigned to birds that showed no head movements (LATHEAD) and no self-righting behavior (end of TI) by the end of the test period. To ensure the continued capturing of untested chicks from their brooding environment, tested chicks were housed elsewhere.

The above experimental procedures were duplicated at 15 d of age which served as an experimental replication (i.e., an additional 80 untested chicks, 20 birds per stress line*implantation treatment were TI tested). In order to minimize separation distress during testing on each day of the study, approximately only 10% of the commingled representatives of each treatment combination housed in a brooding area were tested daily.

3.2.4 Statistical Analyses

INDS, LATHEAD, and TI data were subjected to nonparametric randomized block ANOVAs that incorporated 2 x 2 factorial arrangements of treatments. The factorial was made on the effects of stress line (LS vs. HS) and maternal implantation treatment (CON vs B-implant). The blocks or “experimental replications” were made on the two consecutive days of observation (14 and 15 d of age for TI tests). Duncan’s (DNMRT) was used to partition line*implantation treatment interaction differences in mean IND, LATHEAD, TI responses.

3.3 Results

The HS chicks required fewer ($P < 0.0005$) INDS to achieve TI than did the LS ones (Fig. 1, top panel). However, maternal implantation treatment did not alter INDS (Fig.1, middle panel) and post-hoc partitioning of the line by implantation treatment interactive effects (Fig. 1, bottom panel) showed that both HS-CON and HS-B-implant treatments required similar and fewer ($P < 0.01$) numbers of INDS than did either of the two similarly responding LS treatments. On average, the LS chicks also took less ($P < 0.02$) time to show their first alert head movement (LATHEAD) after successful induction into TI than did the HS chicks (Fig. 2, top panel), but

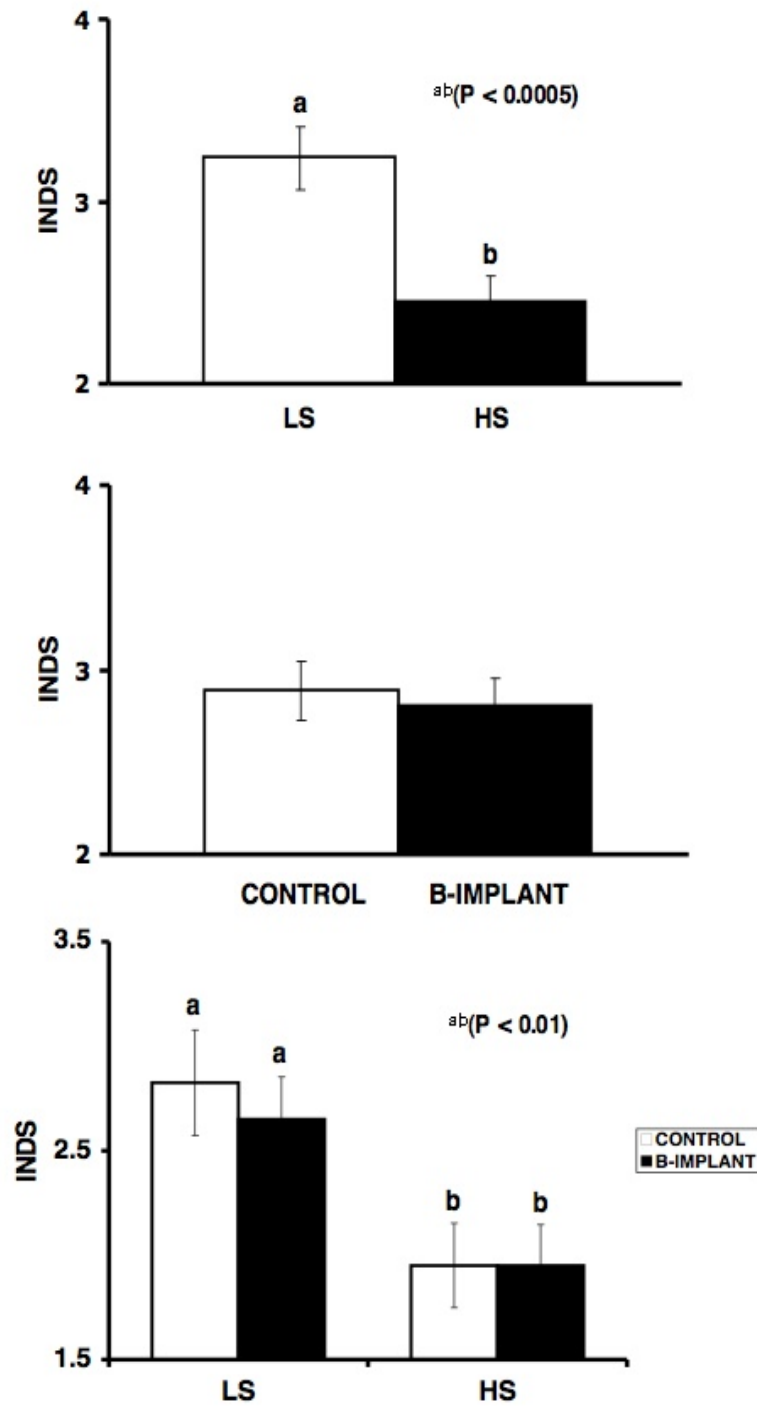


Figure 1. Stress line (top panel), implantation treatment (middle panel) and their interactive effects (bottom panel) on mean (\pm SE; vertical bars) numbers of inductions (INDS) needed to achieve tonic immobility in 14-15 d-old offspring of the implanted hens.

neither the effect of maternal implantation treatment (Fig. 2, middle panel) nor its interaction with stress line (Fig. 2, bottom panel) were effective in altering LATHEAD. Mean durations of TI were unaffected by line, implantation treatment, or their interaction (Fig. 3).

3.4 Discussion

Fewer INDS attempts to achieve TI were needed for HS than LS chicks which agrees with previous reports (Jones et al., 1992b; Satterlee et al., 1993) that HS quail are more susceptible to induction into TI. A high susceptibility to induction into TI may be helpful to ward off predators since the purpose of exhibiting TI behavior per se (the purportedly final stage of anti-predator behavior believed to progress from freezing to fight to flight to immobility; Ratner, 1967) is to reduce predator interest in prey (Gallup, 1977; Jones, 1986, 1987b; Boissy, 1995; Korte, 2001). Thus, birds requiring fewer INDS into TI are thought to be more fearful (Gallup, 1977; Boissy, 1995; Jones, 1996; Cockrem, 2007). Because TI is also thought to be an innate (unlearned) behavior (Gallup, 1977) that reflects underlying fearfulness (Jones, 1996), it is not surprising to see this stress line (HS > LS) INDS result occurring repeatedly in lieu of the proposed connection between heightened adrenocortical activity and increased fear in these lines (Jones et al., 1992ab, 1994b, 1999; Satterlee et al., 1993; Jones and Satterlee, 1996) and in other studies (Jones et al., 1988; Boissy, 1995; Fraisse and Cockrem, 2006; Cockrem, 2007). The reader is reminded here that both unstressed and stressed HS quail hens are also known to secrete higher levels of B into their egg yolks than do LS hens (Hayward et al., 2005) and that B challenge of unselected quail hens during egg formation results in heightened HPA responsiveness to brief restraint (a trait in common with HS quail) in adult offspring hatched from B-treated mothers (Hayward and Wingfield, 2004).

Herein, LS chicks again showed shorter LATHEAD behavior once inducted into TI than did HS chicks as previously observed (see Jones et al., 1992b; Satterlee et al., 1993). Because

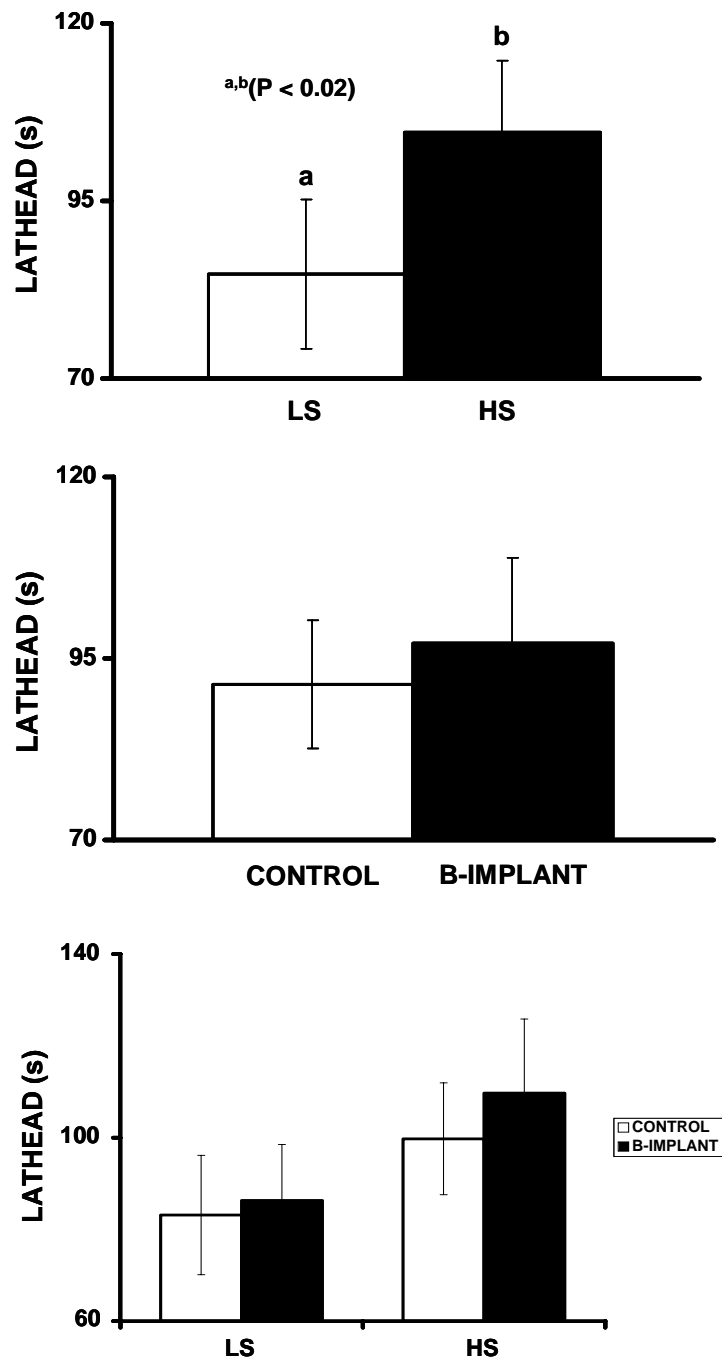


Figure 2. Stress line (top panel), implantation treatment (middle panel) and their interactive effects (bottom panel) on mean (\pm SE; vertical bars) latency to first alert head movement (LATHEAD) during tonic immobility in 14-15 d-old offspring of the implanted hens.

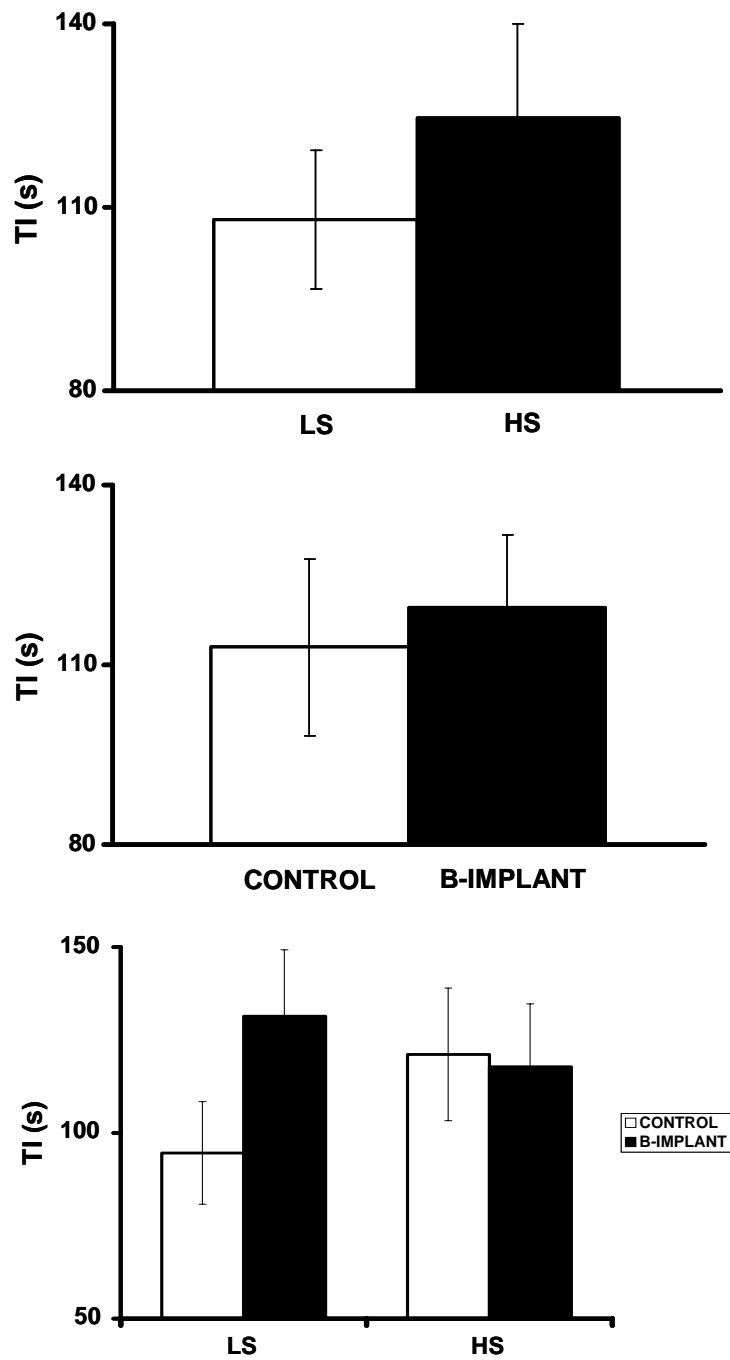


Figure 3. Stress line (top panel), implantation treatment (middle panel), and their interactive effects (bottom panel) on mean (\pm SE; vertical bars) durations of tonic immobility (TI) in 14-15 d-old offspring of the implanted hens.

one of the first actions a bird may take before righting itself from TI is that of an alert lifting of its head (Jones, 1986), it follows that the novel TI test conditions and/or the experimenter are likely being perceived as less of a threat by LS than HS chicks. The idea that LS chicks may perceive humans as less of a threat has been proposed by Jones et al. (1994b), who demonstrated LS chicks show less avoidance of the faces of both a familiar caretaker and an unfamiliar experimenter than do HS chicks. Furthermore, when compared to HS quail, LS quail also show less avoidance of a novel object (a multicolored fishing float placed in their feed troughs; Satterlee and Jones, 1999, Unpublished findings). Consistent with the anti-predator (fear) TI hypothesis, avians consider natural and simulated natural predators (e.g., stuffed hawks), humans, artificial eyes, and even their own mirror reflections as predators in exhibiting TI behavior (Gallup, 1977).

Because of the line differences found in the INDS ($LS > HS$) and LATHEAD ($LS < HS$) behaviors, the duration of TI was expected to be reduced in LS quail as well. However, although HS quail remained in TI on average about 16% longer than LS quail, this difference was only numerically and not statistically relevant. The present lack of a significant line difference in the duration of TI contrasts with previous studies that found a longer duration of TI in HS than LS quail of a similar age (Jones et al., 1992b; Satterlee et al., 1993). This discrepancy cannot be readily explained. However, when measuring fearfulness, certain fear-related variable results may not be consistently found between studies that have B treatments in common. Indeed, Cockrem's (2007) review on the interrelationships between stress, B, and avian personalities concludes that "although activation of the HPA axis when an animal perceives a stimulus to be a threat is considered to occur simultaneously with the basic emotion of fear" it can be challenging to relate fearfulness to B responses since oftentimes "individual measures [of fear behavior] are not sufficient to quantify fear in birds." In other words, fear may not always be accurately

measured by assessment of a single fear-related variable or test of fear. In fact, Cockrem (2007), in reviewing one of his own studies (Fraisie and Cockrem, 2006), wherein the duration of individual TI measures to handling-induced B responses in the same chickens could not be linked, found that, by combining observations from four components of TI testing (INDS, LATHEAD, numbers of alert head movements, and duration of TI) into a “fear score rank” index, a significant correlation between this index and B responses resulted. This helps explain why the relationship between adrenocortical activation and heightened fearfulness in the quail stress lines (Jones et al., 1988, 1992ab, 1994b, 1999; Satterlee et al., 1993; Jones and Satterlee, 1996) is not always straightforward. Nevertheless, the present TI findings (considering that 2 of the 3 TI behavior variables measured showed line differences) do little to change the original contention (Jones, 1987b, 1996) that fear and distress (adrenocortical activation) are positively correlated.

New to the present experiment is the assessment of influences of B-implant treatments in LS and HS hens during egg formation on their offspring’s TI behaviors. Surprisingly, neither maternal B-implantation treatment nor its interaction with stress line affected INDS, LATHEAD, or the duration of TI. These findings indicate that exogenous B treatment of stress line hens does not further alter the line differences in fear ($HS > LS$) detected in previous studies (Jones et al., 1992b; Satterlee et al., 1993) and herein. These results were unexpected for the following reasons. Firstly, B-implant treatment in genetically unremarkable quail hens (Hayward and Wingfield, 2004), a treatment identical to that used herein, is known to increase *in ovo* levels of B and heighten HPA responsiveness to brief immobilization in adult offspring of B-treated mothers. Moreover, the restraint stressor used by Hayward and Wingfield (2004) is the same stressor used to genetically select the LS and HS lines. Thus, it was felt that, when compared to progeny of LS-CON hens, the offspring of B-implanted LS mothers would be prime candidates

for maternal B-induced conversion into animals that would express greater adrenal responsiveness to stress and therefore be more fearful, attributes present as a result of genetic selection in the HS quail. It was further reasoned that B-treatment of HS hens may or may not further exacerbate the stress*fear relationship in their HS chicks, depending upon whether selection for exaggerated adrenocortical responsiveness in the HS line has been maximized or not. Pre-experimental expectations were also reasonable considering the report in chickens (Janczak et al., 2006) that fear (avoidance of humans) is increased in chicks hatched from eggs injected with B.

Thus, the question remains: how does one explain the present lack of maternal B-implant influences on TI behaviors? It may be that genetic selection for divergent B response to stress has altered the quail lines' genomic controls of adrenocortical responsiveness in such ways that the HPA axis and therefore fear activity of stress line progeny cannot be further altered by whatever yet unidentified mechanism(s) that additional *in ovo* B during embryogenesis apparently uses to alter HPA activity (Hayward and Wingfield, 2004) and fear behavior (Janczak et al., 2006) in unselected avians. That said, however, it is important to note that Janczak et al. (2007a) have also found that, while Leghorn hens stressed by feed restriction secreted more fecal B metabolites, levels of B in the albumen and yolks of their eggs were unaffected, yet the adult progeny of these stressed hens still showed longer durations of TI. This suggests that altered fear responsiveness in the offspring of these stressed mothers was the result of some other mechanism(s) independent of *in ovo* B. In yet a third study, Janczak et al. (2007b) found *in ovo* B injections during embryogenesis ineffective in altering TI responses in 4-wk-old chicks hatched from the B-treated eggs. Rubolini et al. (2005) have also found no changes in TI responses of yellow-legged gull chicks hatched from B-injected eggs. Thus, the three Janczak chicken studies (2006, 2007a, b) and the gull study of Rubolini et al. (2005) present an unclear

picture of the relationship between maternal and *in ovo* B influences on altering the TI behavior of avian offspring. Like the literature proposed relationship between increased adrenal stress responsiveness and heightened fear, the avian maternal (or *in ovo*) B*offspring fear relationship is apparently also not straightforward and it awaits further clarification. That said, it should also be noted here that the HWB studies (see Chapter 4), which were conducted with a different and slightly older but full sibling group of birds to those presently tested for TI responses, showed the latency times of both head and full body emergence from the HWB were stymied, regardless of line, in the offspring of hens implanted with B. These HWB emergence findings lend support to the report by Janczak et al. (2006) that chicks hatched from eggs injected with B show greater fear of humans and they also argue in support of a transovarian link between B and heightened fear as measured by offspring inactivity.

CHAPTER 4

HOLE-IN-THE-WALL BOX EMERGENCE RESPONSES IN OFFSPRING OF JAPANESE QUAIL STRESS LINES TREATED WITH CORTICOSTERONE DURING EGG FORMATION

4.1 Introduction

The HWB emergence test is used to study the timidity aspect of fear in poultry (Jones, 1987b). Birds that take longer to emerge from a “safer” dark compartment into a lighted novel (presumably more frightening) area are deemed to be more fearful (Jones, 1987b; Jones, 1996). Vocalization responses are also sometimes measured during the acclimation period to the dark compartment of the HWB. Vocalization outcomes (either exacerbation or inhibition of “talking”) in this and other tests that assess fearfulness have been interpreted differently in terms of fear measurement depending on the intensity of the fear-eliciting stimulus and length of fear behavior observation because of the proposed progressive nature of fear states (Jones, 1987b).

Previous studies conducted that involved measurement of HWB responses of Japanese quail from the LS and HS lines showed that LS chicks emerge sooner from the dark compartment of the HWB than do their HS counterparts (Satterlee and Jones, 1995; Jones et al., 1999). Also, more LS chicks vocalized while acclimating to the dark compartment of the HWB than did HS chicks in the study performed by Satterlee and Jones (1995).

Non-selected quail hens implanted with B during egg formation have increased levels of plasma B associated with greater deposition of B into their egg yolks (Hayward and Wingfield, 2004). Such treatment dampens early growth rates of chicks and enhances stressor-induced sensitivity of the HPA axis in adult progeny of B-treated hens. Chicks hatched from *in ovo* B treatments also show a reduced food drive and more fear of humans (Janczak et al., 2006). Hayward et al. (2005) also found both unstressed and stressed HS hens to deposit more B into their egg yolks than do LS hens. Therefore, the present study was conducted to examine the dark

compartment HWB acclimation vocalization and HWB emergence fear responses of juvenile offspring from LS and HS hens fitted with empty (control) or B-filled implants during egg formation.

4.2 Materials AND Methods

4.2.1 Genetic Stocks and Animal Husbandry

Offspring from generation (G)₃₈ of two lines selected for either low (low stress, LS) or high (high stress, HS) plasma B response to brief immobilization were studied. Satterlee and Johnson (1988) have described the genetics that underlie the first 12 generations of pedigree selection and the most recent genetic history of the lines, up to G₃₄, is discussed in detail elsewhere (Satterlee et al., 2000; Marin and Satterlee, 2004; Satterlee et al., 2006). Although line differences in levels of plasma B were not measured in the present study, recent findings in the stress lines attest to the maintenance of divergent adrenocortical responsiveness to a variety of non-specific systemic stressors. Indeed, Satterlee et al. (2007) have most recently offered explanations as to why the gene(s) that control the adrenocortical responsiveness trait in these lines have likely become fixed.

Ninety-six hens from each line (48 LS + 48 HS) were used. At 29 wk of age, each hen was pair housed with a non-sibling, same-line male in a single cage of one of two Alternative Cage Designs (Alternative Design Manufacturing and Supply, Inc., Siloam Springs, AR) four-tier cage batteries. Each battery contained 48 pedigree-style breeder cages (individual cage dimensions were 50.8 x 15.2 x 26.7 cm, length x width x height, respectively). Care was taken to insure that each of the breeding pairs selected, while randomly selected from larger family populations within each line of the same hatch, constituted, as nearly as possible, equal representation of the 12 different families that make up each line. A breeder ration (21% CP; 2,750 kcal ME/kg) and water was provided to the birds *ad libitum*. The daily photostimulatory

cycle was 14 L: 10 D (approximately 280 lux during the lighted portion of the day); lights-on was at 6:00 h and lights-off was at 20:00 h daily. Daily maintenance and feeding chores were done at 8:00 h daily.

4.2.2 Hen Treatments

At 33 wk of age, half of the hens from each line ($n = 24$ birds/line) were individually fitted with 16 mm silastic-tube (Dow Corning, Midland, MI; Cat. No. 508-006) implants containing either corticosterone (B; Sigma–Aldrich Co., Atlanta, GA; Cat. No. C2505) or no B (controls, CON). Implants were placed s.c. in the back of the neck using a No. 10 biopsy needle (Becton Dickinson, Franklin Lakes, NJ). The implant tubes were sealed at one end with silicone sealant and open on the other end. Hens were allowed a 10 d acclimation period to allow sufficient time for maternal B deposition into the eggs of B-treated hens (Hayward and Wingfield, 2004) to their implantation treatments. Eggs were then collected daily, identified by pencil markings as to their origin by hen line and implantation treatment, and stored at 18 C until incubation. Egg collection lasted for 3 wk and these eggs were then set together into an incubator (NatureForm NMC 2000; NatureForm Hatchery Systems, Jacksonville, FL). During the first 14 days of incubation, eggs were turned 6 times a day and subjected to 37.5 C and 62% RH. Upon transfer of the eggs to a second NMC 2000 hatcher unit on Day 14, eggs were no longer turned and incubation conditions were changed to 37.2 F and 69% RH.

4.2.3 Offspring and Variables Measured

At hatch, chicks were leg banded with appropriate different color and uniquely numbered leg bands that allowed their identification with the four line*implantation treatments (LS-CON, LS-B-implant, HS-CON, and HS-B-implant). Chicks were brooded, all treatments equally co-mingled, in three confinement rings (approximately 260 chicks/ring). This arrangement resulted in about 65 chicks from each treatment combination being represented in each ring. The

brooding ring areas were of identical construction- each ring was 1.2 m in diameter, heated with two 125-watt incandescent lamps, and had pine wood shavings as a floor substrate. Chicks were fed a quail starter ration (28% CP; 2800 Kcal ME/kg) and given water *ad libitum*. Brooding temperatures and their change with time were similar to those used by Jones and Satterlee (1996).

At 21 d of age, 80 chicks (20 each from the LS-CON, LS-B, HS-CON, and HS-B treatment groups not previously tested in the TI studies conducted at 14 and 15 d of age) were randomly selected for HWB studies. Individuals were randomly captured throughout the test day in equal rotation from each of the three confinement rings until the above sample numbers were achieved. Upon capture, a test chick was removed to a separate room (i.e., the HWB test apparatus was located in a quiet area, approximately 13 m away from the live-bird facility, and free from bird noises and human traffic) and its HWB responses were measured as follows. The testing box had two compartments (one dark and one lighted) measuring 21 x 21 x 21 cm (length x width x height). The dark compartment was constructed of aluminum with a 1-cm wire mesh floor and the lighted compartment was made entirely of wire mesh. Separating the two compartments was an aluminum wall with a 10 x 8-cm hole (height x width) covered by a guillotine trap door. The birds were placed individually into the dark compartment and given a 1 min acclimation period after which the guillotine door was raised.

The number of chicks that vocalized as a proportion of the total number of chicks tested (PVOCS), the latency to first vocalization (LATVOC; s) and the number of vocalizations (VOCS) before the guillotine door was raised were recorded. Chicks that did not vocalize during the 60 s acclimation period were given scores of “60” for LATVOC and “0” for VOCS. The number of chicks that vocalized during the 1 min acclimation period in the dark box as a proportion of total number of chicks tested in a line*implantation treatment combination

(PVOCS) were also determined. The latencies from raising the door to head emergence through the hole in the wall of the dark box (head emergence; HE, s) and complete body emergence into the lighted compartment (full emergence; FE, s) were also recorded. Using a test ceiling of 10 min, maximum scores of 600 s were given to a chick that did not exhibit HE or FE behavior. Tested birds were rehoused in an area separate from their home-brooding area to ensure the capture and HWB testing of only untested chicks.

An experimental replication was performed at 23 d of age (i.e., the HWB responses of an additional 80 untested chicks, 20 birds per stress line*implantation treatment were determined). In order to minimize separation distress during HWB testing on each day of the study, approximately only 10% of the commingled representatives of each treatment combination housed in a brooding area were tested daily.

4.2.4 Statistical Analyses

PVOCS, LATVOC, VOCS, HE, and FE data were subjected to nonparametric randomized block ANOVAs that incorporated 2 x 2 factorial arrangements of treatments. The factorial was made on the effects of stress line (LS vs. HS) and maternal implantation treatment (CON vs B-implant). The blocks or “experimental replications” were made on the two consecutive days of observation (21 and 23 d of age for HWB test). Duncan’s (DNMRT) was used to partition line*implantation treatment interaction differences in mean LATVOC, VOCS, HE, and FE responses. The PVOCS variable is a binary trait (i.e., chicks either vocalized or not); therefore, a standard proportion test of differences was used for this variable.

4.3 Results

During the acclimation period in the hole in the wall box dark compartment, on average, the number of chicks that vocalized as a proportion of the total number tested (PVOCS) was much greater ($P < 0.0001$) for chicks of the HS line (Figure 4, top panel). The HS chicks also

vocalized much sooner ($P < 0.0001$; LATVOC, Figure 5, top panel) and much more often ($P < 0.0001$; VOCS, Figure 6, top panel) than did the LS chicks. And, while maternal implantation treatment did not affect PVOCS (Figure 4, middle panel) or LATVOC (Figure 5, middle panel), CON chicks tended to vocalize more ($P < 0.07$) than did B-implanted ones (VOCS, Figure 6, middle panel). The stress line by implantation interactive effects on mean PVOCS and LATVOC were non-significant and the Duncan's analyses for these effect's mean responses (Figures 4 and 5, bottom panels, both $P < 0.01$) simply reflected the main effect of stress line (i.e., more HS chicks vocalized sooner than LS ones regardless of implantation treatment). However, a line*implantation treatment interaction ($P < 0.02$) was found for VOCS. Post-hoc partitioning of the interactive VOCS means showed that the HS-CON chicks vocalized more ($P < 0.01$) than the other three similarly less vocal groups (Figure 6, bottom panel).

Although stress line did not affect HE or FE (top panels of Figures 7 and 8, respectively) into the lighted compartment of the HWB, mean HE (Figure 7, middle panel) and FE (Figure 8, middle panel) occurred sooner ($P < 0.06$ and $P < 0.05$, respectively) in the CONs than in chicks hatched from B-implanted hens. The line and implantation treatment showed no interaction in affecting HE and FE (bottom panels of Figures 7 and 8, respectively).

4.4 Discussion

During the acclimation period to the dark compartment of the HWB, PVOCS were dramatically higher in HS than LS quail, and HS chicks also showed markedly reduced LATVOCs and greater VOCS than LS chicks. Line differences in the LATVOC and VOCS behaviors have not been assessed previously, but the present PVOC result contrasts with an earlier study wherein more LS chicks vocalized than did HS ones during HWB acclimation (Jones et al., 1999). In that study, it was concluded that the line difference in the number of chicks that vocalized during HWB acclimation was indicative of and consistent with the lower

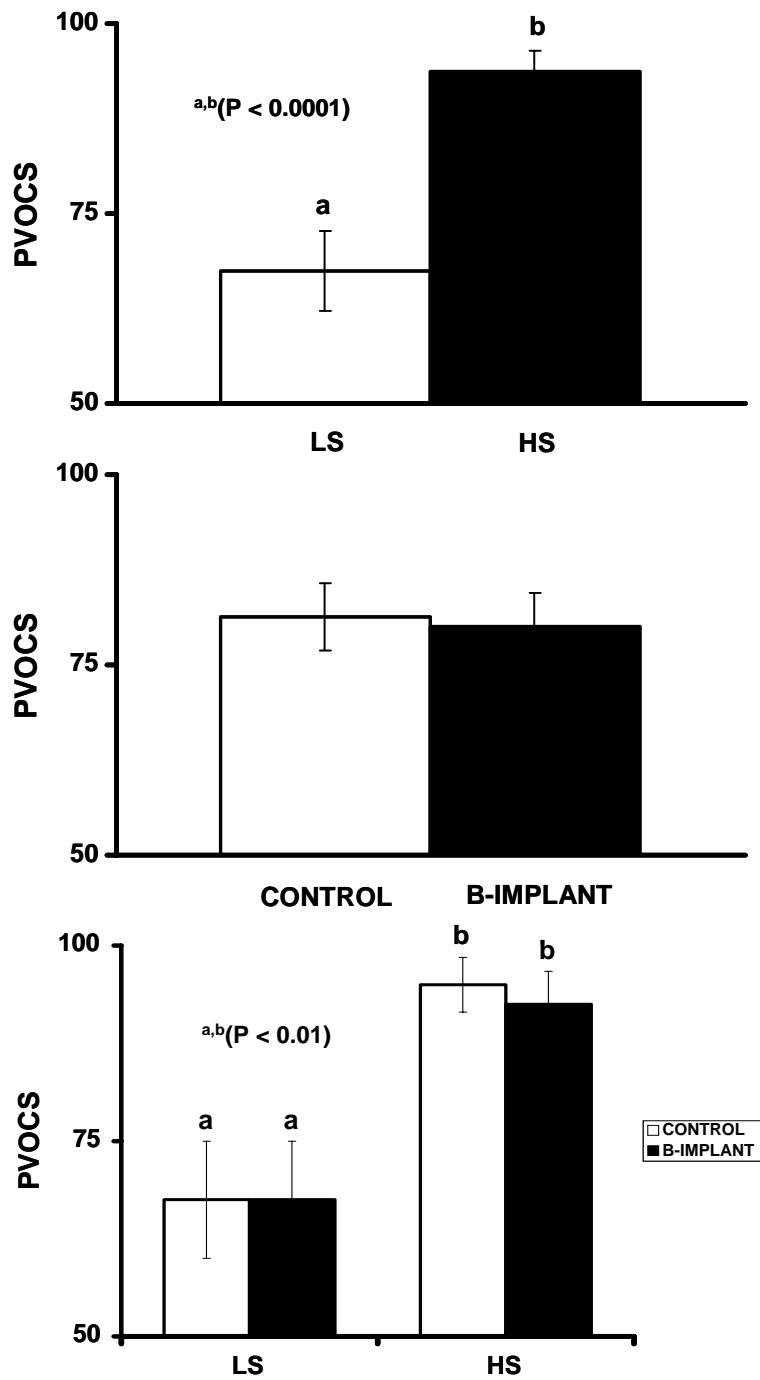


Figure 4. Stress line (top panel), implantation treatment (middle panel), and their interactive effects (bottom panel) on mean (\pm SE; vertical bars) numbers of chicks that vocalized as a proportion of the total number tested (PVOCS) in the dark compartment of a hole-in-the-wall box in 21-23 d-old offspring of the implanted hens.

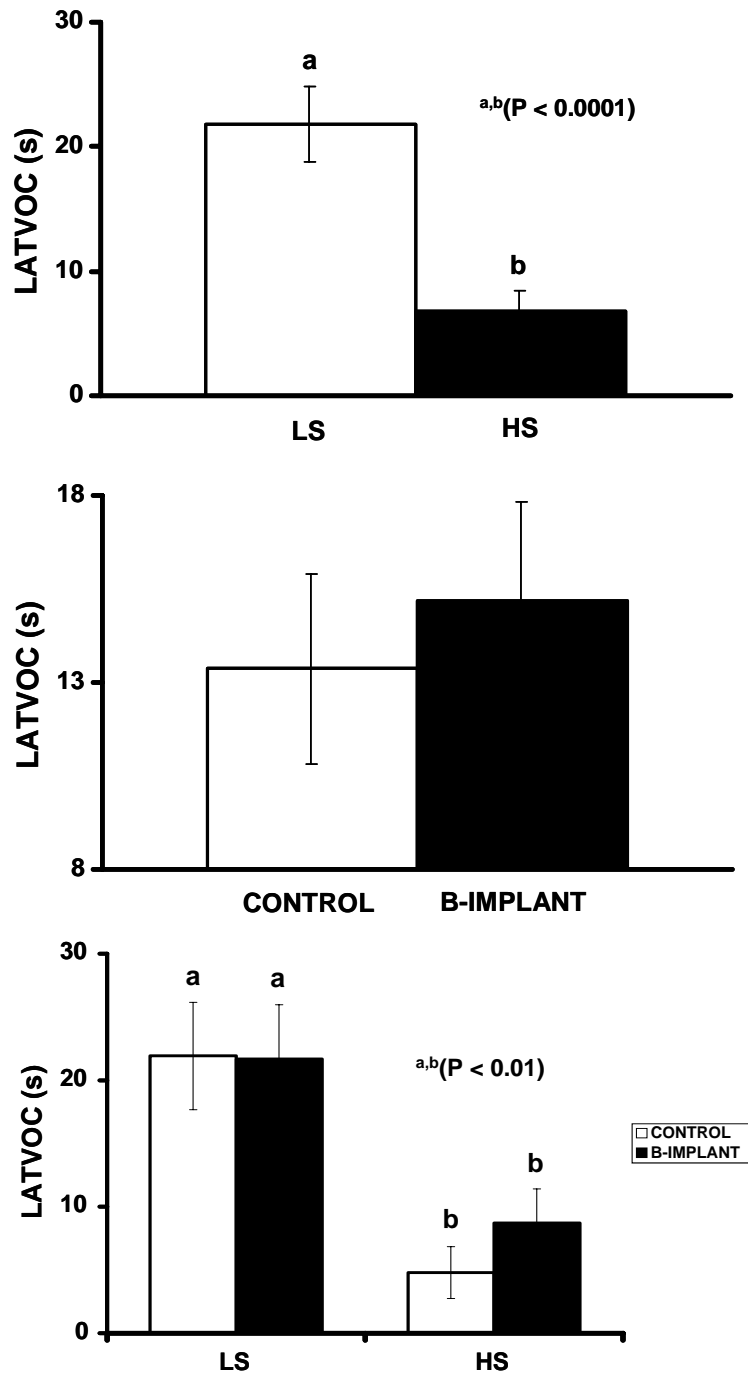


Figure 5. Stress line (top panel), implantation treatment (middle panel), and their interactive effects (bottom panel) on mean (\pm SE; vertical bars) latency to vocalize (LATVOC) in the dark compartment of a hole-in-the-wall box in 21-23 d-old offspring of the implanted hens.

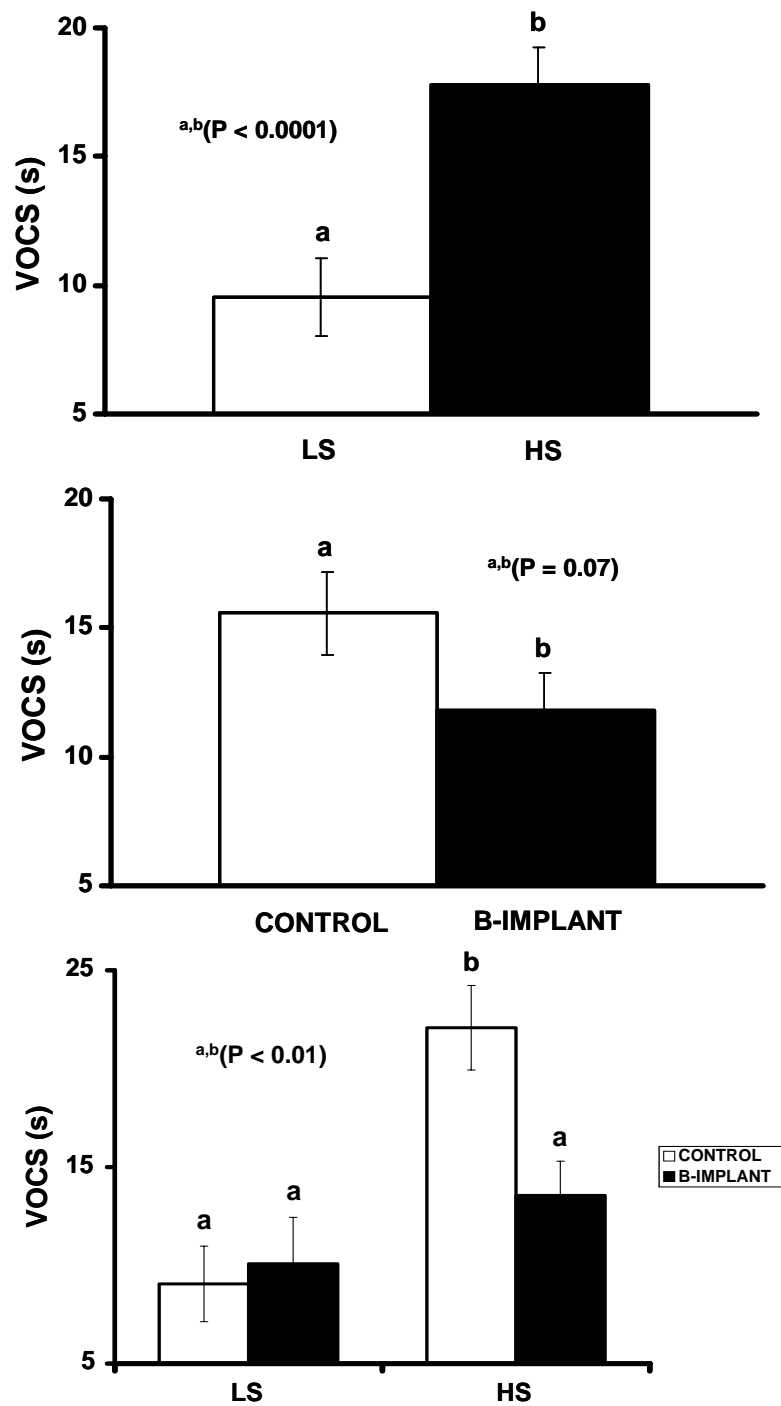


Figure 6. Stress line (top panel), implantation treatment (middle panel), and their interactive effects (bottom panel) on mean (\pm SE; vertical bars) numbers of vocalizations (VOCS) in the dark compartment of a hole-in-the-wall box in 21-23 d-old offspring of the implanted hens.

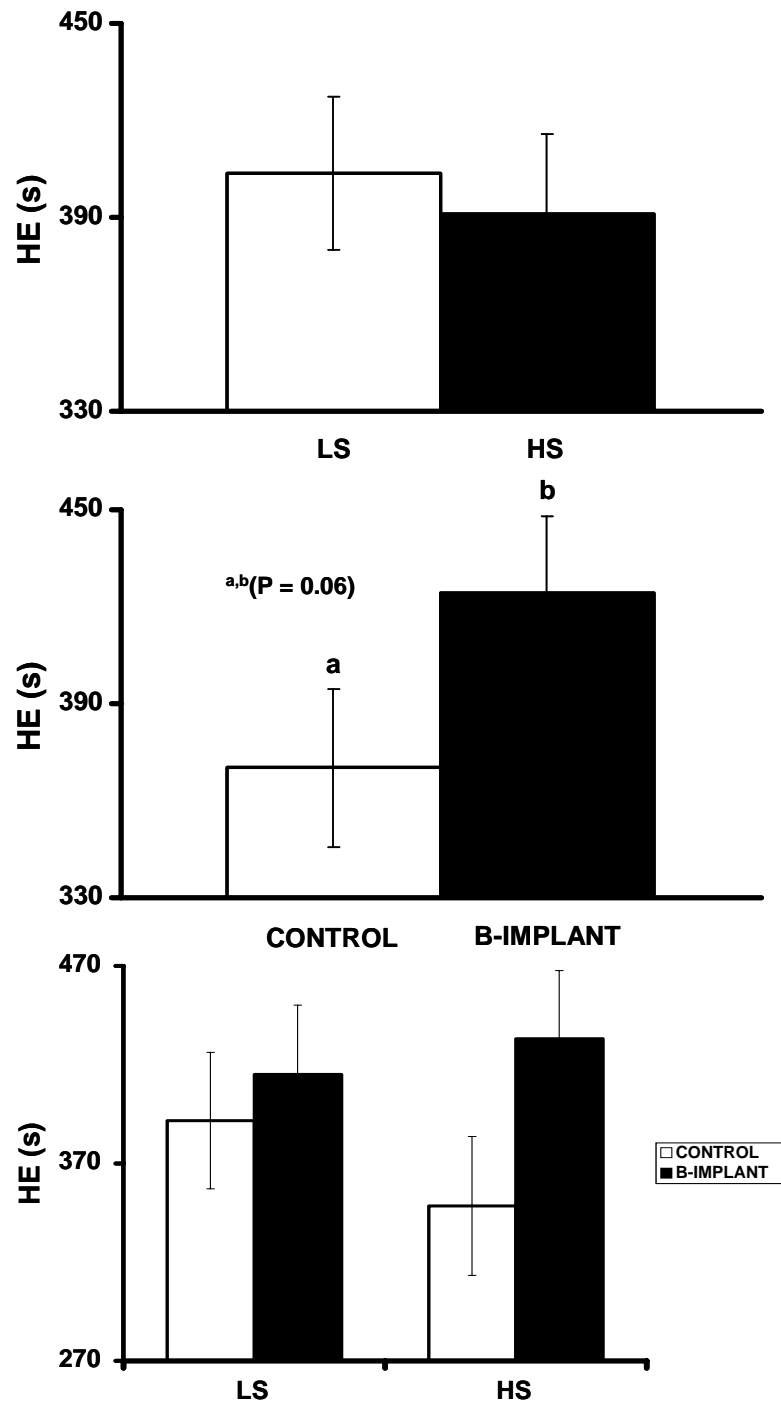


Figure 7. Stress line (top panel), implantation treatment (middle panel), and their interactive effects (bottom panel) on mean (\pm SE; vertical bars) head emergence (HE) from a hole-in-the-wall box in 21-23 d-old offspring of the implanted hens.

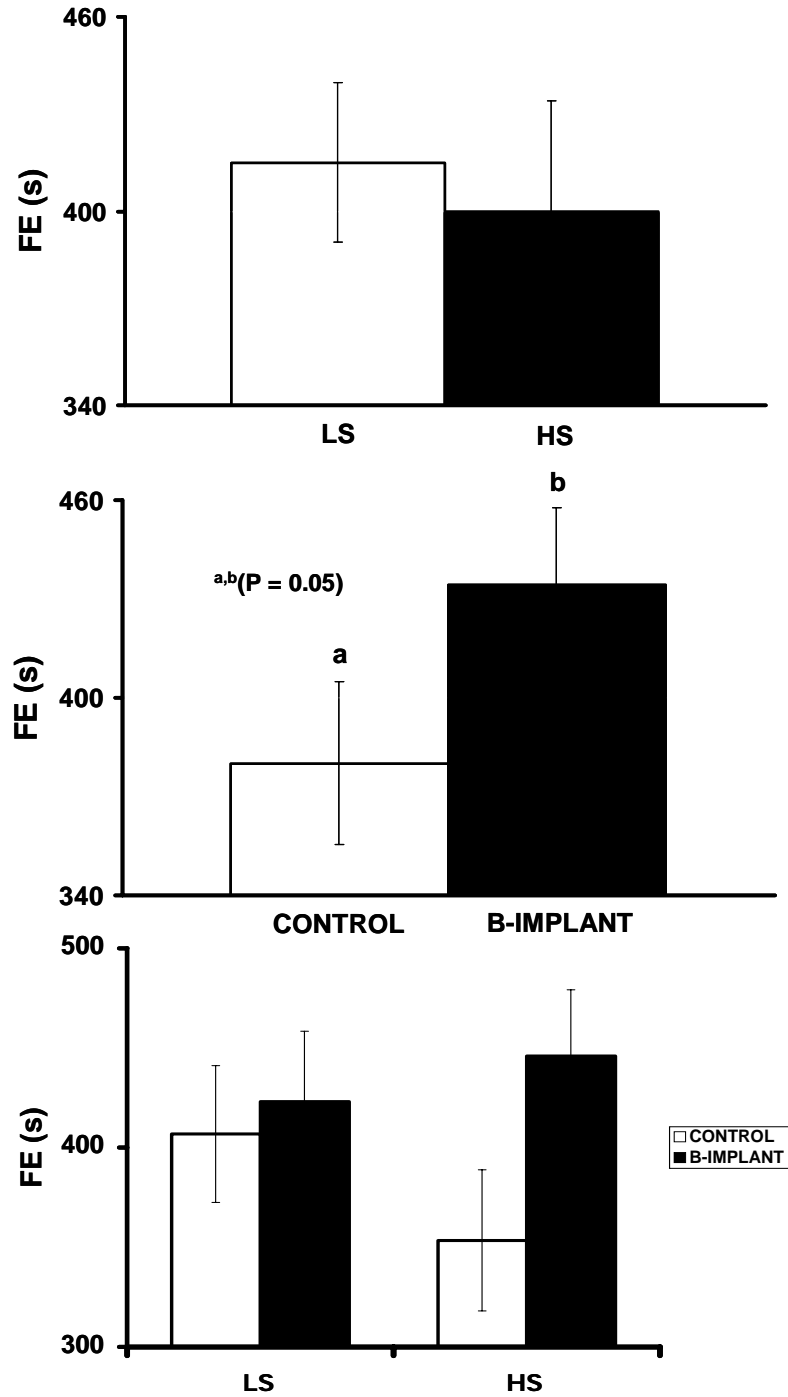


Figure 8. Stress line (top panel), implantation treatment (middle panel), and their interactive effects (bottom panel) on mean (\pm SE; vertical bars) full body emergence (FE) from a hole-in-the-wall box in 21-23 d-old offspring of the implanted hens.

fear reactions (i.e., sooner HE and FE from the HWB once the guillotine door was raised allowing access to a presumably more frightening unfamiliar, lighted space) also observed therein. These findings were all consistent with the reduced fear reactions of LS quail found in numerous forerunner studies using other measurements of fear (e.g., open field behaviors, Jones et al., 1992a; TI, Jones et al., 1992b, Satterlee et al., 1993; avoidance of humans, Jones et al., 1994b; and, struggling in a crush cage, Jones and Satterlee, 1996).

However, the ‘silence element’ of the fear hypothesis in HWB testing has not been just one sided. For example, Leghorns deemed to be “docile” (assumedly less fearful birds) had a shorter LATVOC in emergence testing than did their “flighty” counterparts (Jones and Mills, 1983). Indeed, most would agree it is, at best, difficult for humans to know exactly what birds are “saying” when they vocalize and, admittedly, this author is not expert in interpreting quail speak. However, reviews by Jones (1987b, 1996) may be helpful here. They suggest that fear can either elicit or inhibit different numbers and qualities of vocalizations in avians depending upon the degree of novelty of environmental stressors as they relate to levels of fear. For example, low levels of novelty and fear have been associated with peeping distress calls while intermediate fear levels often induce high–intensity peeping and high levels of fearfulness may suppress vocalizations. Satterlee (personal communication) has the distinct impression that the vocalizations previously measured in the stress lines (Jones et al., 1999) were soft, infrequent, and only seen in a few birds. In fact, 59 of the 80 earlier tested quail did not vocalize at all (see Jones et al., 1999). These former vocalization findings are quite different than those presently observed in that the present vocalizations were very frequent, high-pitched, more harsh and of a louder nature, i.e. more indicative of “alarm calling” (see below). The lack of much calling and the gentle nature of the VOCS of the few birds that called in the previous study (Jones et al., 1999) suggests that the fear levels associated with HWB testing produced then were likely much

greater than what occurred in the present study. This may help explain why the line differences in PVOCs contrasted between the 1999 (LS > HS) and present (HS > LS) studies. Indeed, Boissy's (1995) review concludes that many avian species (e.g., magpies and domestic fowl) vocalize in the form of what has been called "alarm calls" when they detect predators. He defines alarm calls as signals or sounds whose structure can be varied in a graded fashion and communicates possibilities of danger to conspecifics. Such calling is believed to be influenced by physical characteristics of a frightening stimulus that is perceived to be predatory— factors such as stimulus presentation, movement, intensity duration, suddenness, or proximity. For example, Evans and Marler (1991) found the velocity of flying hawks in video images and image manipulation of visual distances from this known chicken predator were crucial in eliciting alarm calls in test (prey) chickens. Boissy (1995) has also suggested that novelty (such as bird capture by the experimenter, transport to the HWB apparatus, separation from live conspecifics, and placement into the dark compartment of the HWB were possible novel situations for the test birds of the present experiment) is "one of the most potent experimental conditions" that can lead to negative emotional responses, such as alarm calling. Moreover, exposure of chicks to alarm calls recorded from conspecifics exposed to a predator made them peep and run away, whether they had previously encountered a predator or not (Duncan and Filshie, 1979). Therefore, it is possible the present increased PVOCS, decreased LATVOC, and increased VOCS in HS quail are a simple reflection of greater anti-predatory behavior of heightened alarm calling. In other words, the HS quail may have perceived the nature of the cascade of events from human bird capture to placement in the dark compartment of HWB to be a more predacious experience than did LS quail. It is unfortunate that the vocalizations of LS and HS quail were not recorded so that they could be used in audio play back studies that would assess differences in the observed line

vocalizations on peeping and run away behaviors (see HE and FE discussion below) of unselected quail.

It is also important to note here that, since the earlier report of Jones et al. (1999), *in ovo* B-treatment was found to increase the number and intensity of VOCS in yellow-legged gull hatchlings (Rubolini et al., 2005) and the numbers of “distress vocalizations” in domestic chicks following “release into a novel arena” (Freire et al., 2006). In addition, Vierin and Bouissou (2003) have used the utterance of more frequent high-pitched bleats to judge levels of fear in lambs in distress. Ultrasonic and audible fear-induced alarm call responses have also been documented in rats (assumed to be distress calls related to anxiety; Kikusui et al., 2001, 2003), squirrel monkeys (McCowan et al., 2001), and ground squirrels (Wilson and Hare, 2004).

For the most part, B-treatment of LS or HS hens did not alter their respective offspring’s vocalization activity beyond the effects of stress line genome *per se* as discussed above. However, hen B-implantation treatment did interact with line in affecting the VOCS variable of progeny. Specifically, birds of the HS-CON group showed more VOCS than the other three treatment groups. These results lead to a conclusion that maternal B-treatment is capable of decreasing the number of vocalizations in HS but not LS quail offspring. Thus, maternal B-treatment of hens genetically predisposed towards exaggerated adrenocortical responsiveness (i.e., the HS hens) may result in a shift of their offspring’s vocalization fear responses more quickly away from alarm calling (which is linked to lower levels of fear) to silence (which is associated with the fourth stage and highest level of fear) as postulated by Ratner (1967) and Jones (1987, 1996).

Stress line did not significantly affect the times of HE or FE from the HWB. These results were also unexpected as they did not confirm previous studies (Satterlee and Jones, 1995; Jones et al., 1999) that found LS quail emerged into the unfamiliar and lighted compartment of

the HWB apparatus sooner than did HS birds. Furthermore, only 7-9 d earlier in the TI testing of full siblings to the presently tested HWB quail, LS quail were found to be more resistant to induction into TI and they had a shorter LATHEAD once successfully induced into TI than did HS quail (see Chapter 3). And, if the robust vocalization line differences that occurred in the dark compartment of the HWB truly reflect greater “alarm calling” in HS than LS quail as has been presently proposed, then it is logical to suspect HS quail to have been more frightened than LS quail and therefore for HS quail to take more time to show HE and FE. It is also widely held that fear is associated with inactivity in birds (see reviews of Jones, 1987b, 1996). In fact, in addition to the previous HWB emergence tests (Satterlee and Jones, 1995, Jones et al., 1999), when various locomotion behaviors were used as assessment elements in other behavioral tests of fear, HS quail have invariably (until now) shown reduced locomotor activity (e.g., various open field behaviors, Jones et al., 1992a; struggling in a crush cage, Jones and Satterlee, 1996, Jones et al., 2000).

While a readily apparent explanation as to why no line differences in HE and FE behavior were presently detected cannot be offered, the times of both HE and FE from the HWB were, however, found to be stymied, regardless of line, in the offspring of hens implanted with B. This is a new and important finding that lends support to the report by Janczak et al. (2006) that chicks hatched from eggs injected with B show greater fear of humans. These latter findings also argue in support of a transovarian link between B and heightened fear as measured by offspring inactivity. On the other hand, considering that B-implant treatment was presently ineffective in altering TI behaviors, and *in lieu* of the controversial literature on maternal/*in ovo*-B influences on altering fear behavior of offspring, the dilemma remains that these relationships are not straightforward and may or may not be dependent on *in ovo* B intervention.

CHAPTER 5

SUMMARY AND CONCLUSIONS

The present studies were conducted to determine fear responses in juvenile offspring of Japanese quail hens selected for divergent adrenocortical stress responsiveness and treated during egg formation with silastic implants that were either empty (controls) or filled with corticosterone (B). The studies are important in that they address the potential interactive influences that quail stress response genome might have with maternal B treatment as such treatments may affect the fear behavior of progeny. Fear and distress are known to have many detrimental consequences on poultry production and animal welfare.

It was concluded that both genetic selection for contrasting adrenocortical stress responsiveness and supplemental B during egg formation can alter fear behavior in quail offspring. However, quail stress line genome may be affecting certain fear and alarm responses in chicks via the same or a different mechanism that underlies how elevating maternal B increases *in ovo* levels of B that in turn alters the fear behavior of progeny. Additional research will be needed to help clarify these issues.

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