The effects of early stress on life-time strategies of behaviour and coping in chickens (*Gallus gallus*)

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1 Abstract
Stress is often an important consideration for animal welfare. A number of factors can contribute to stress in domestic animals, most notably those used in food production. We investigated the effects and heritability of stress in domestic chickens (*Gallus gallus*). Using a spatial learning paradigm, we tested an early social isolation-stressed group and their offspring against unstressed controls, to determine if this cognitive function was negatively affected by stress. In the parental generation, we found that across sessions control birds improved in performance, indicating a learning trend. Stressed birds showed no difference across sessions, indicating a lack of learning. No effects of the parental treatment were found in the offspring of stress and control birds. Social isolation stress was found to affect spatial memory learning, however, we did not find evidence that the parental stress influenced the spatial abilities of the next generation despite changes in other behaviours.

Keywords: Stress, spatial learning, domestic chicken (*Gallus gallus*), heritability

2 Introduction

2.1 Stress
There are many ways of defining stress. Hans Seyle described it as essentially any of a body’s response elicited by a stimuli to which it must adapt (1973). Indeed, most people can recognize that even positive changes can in fact be stressful and require a certain degree of adapting. Walter Canon (1935), succinctly described stress in terms of disruption to homeostasis, which has a particular potency with regards to the physiological aspects of stress. The hypothalamus-pituitary-adreno axis (HPA axis) is the primary organizational and regulatory system in an organism, with respect to stress. This system is the exchange point from which neural messages, integrated from the senses, are translated into endocrinological messages to the rest of the organism. The hormonal aspect of the HPA axis, and therefore the stress response, allows biologists to investigate the internal environment of a living, functional organism by taking blood. The primary stress hormone released by adrenals is cortisol, or its avian equivalent corticosterone. That being said, no hormone acts in complete isolation of others, and so stress effects can also be measured in hormones such as testosterone, which vary in response time to a stressor (hours and days, as opposed to minutes, as with cortisol) (Heiblum et al, 2000).

Just as a number of biological molecules can indicate the presence of stress, a great array of behavioural factors can influence the occurrence and intensity of the perception of a stressor. Forkman et al (2007) investigated the variety of responses observed within a variety of behavioural tests of stress. In the field of avian stress, there are three primary tests of stress, and stress response, which Forkman investigated: novel arena test, novel object test, and the restraint test. While all are designed to test one biologically relevant factor, stress, it was found that across various experiments, the use of various stimuli has an effect on behaviours produced (Forkman et al, 2007). Additionally, Cordero et al (1998) found that the intensity of a stressor can produce a variety of responses. While conditioning rats to a foot shock stressor, corticosterone and fear-related behaviour were related to the intensity of the shock.

Of particular relevance for social species, is the influence of social stress. Apfelbeck and Raess (2008) examined that social isolation stress has apparent negative effects on European starlings (*Sturnus vulgaris*). Two types of behavioural tests were performed, one with visual contact with conspecifics, and one in complete isolation. Birds that were tested in isolation
were found to have higher corticosterone levels, and display more activity. Interestingly, the level of stress due to social isolation was independent of the level of fear towards a novel object, further supporting the notion that different stressors can have varied responses. The presence of social stress in chickens, and the relevance for animal welfare and husbandry practices has been extensively studied (Ghareeb et al, 2008; Guzman and Marin, 2008). For example, Ghareeb et al (2008) found that fearful and social behaviours were consistent over an individual chickens’ lifetime, suggesting that such behavioural measures can be used for selection of stocks more suited to specific housing conditions, decreasing stress and improving overall welfare. Social stressors due to housing conditions (ranging from isolation to over stimulation) are particularly present in most modern chicken farms, where transport and handling can have a further detrimental influence on the welfare over a long time period (El-Lethy et al, 2000; Knowles and Broom, 1990).

Stress can have a negative affect on cognitive processes. Kuzminski et al (2010) found that even a single stressful event can affect the cellular functioning in the rat brain. Evidently, the exposure to stress “primed” the neurons of the brain to further stress events. After a single immobilization stress treatment, particular synapses in the hypothalamus (as a part of the HPA axis) of rats become more likely to fire (i.e. they become more sensitive). Thus, stress not only directly affects the brain, but affects the brain’s future response to stress. Mattson et al (2003) discussed that food stress specifically, increases neural plasticity. That feeding, likely the most essential behaviour to survival, is susceptible to stress, is not surprising. Asthemimer et al (1992) highlighted the relationship between stress and feeding in a study with starlings. To study the effects of corticosterone, exogenous hormone was provided to the test subjects. At first, no differences were noted between the groups (despite elevated serum hormone levels as a result of the treatment). After 24 hours of food deprivation, conversely, the feeding behaviour of the hormone treated group had increased in duration and intensity. Furthermore, hormone treated birds showed greater activity levels and attempted escapes, compared to both controls, and pre-fasting behaviours. From the majority of the examples previously mentioned, the effects of stress had been measured in close temporal proximity to the stressor. In recent years, evidence that stress can have much longer reaching consequences has been accumulating, suggesting that stress cannot just affect immediate behaviours, but have physiological and behavioural consequences later in life, and indeed in later generations (Formanek et al, 2008; Jablonka, 2009; Tchirren et al, 2009).

2.2 Stress During Early Development

Early stress can be defined as the occurrence of a stress event at some stage in development (“early” can essentially mean any time prior to testing, but often means before sexual maturity). The distinction between early stress, and stress is especially important in a learning paradigm, as stressful events after a learning period (not early stress), have been shown to affect memory recall specifically (Kogan and Richter-Levin, 2010). It is important to be aware that stress can have an effect at any point in development, from fertilization onward. For example, it has been shown that growth, corticosterone levels, and hatching success in birds are greatly affected by incubation temperatures prior to hatching (DuRant et al, 2010; Hepp et al, 2006). Considering that the incubation period in birds is often considered analogous with mammalian fetal development, it is unsurprising that similar pre-birth effects of stress have been found in various mammal taxa, including humans (Meek et al, 2000; Schneider et al, 1999; Buitelaar et al, 2003). Some have started to wonder what the earliest stage is in which stress can have an effect. After recognizing that bird phenotype can be influenced by incubation temperatures, it has been investigated to see if stress during egg development could have an effect. And indeed it does. Naguib and Gil (2006) found that the
negative effects of enlarged brood sizes in which a parental bird was raised, resulted in smaller offspring in the following generation, which also had lower reproductive success. They concluded that the effects of stress on the parents were transmitted across generations to the offspring, prolonging the effects of stress. Okuliarova et al, (2010) showed Japanese quail that had been stressed by a restraint had increased corticosterone levels in the yolks of the eggs they laid. This study indicated that maternal hormone levels, affected by a stressor, can influence the developmental environment of the chick embryo, providing a possible mechanism for transmission of epigenetic stress effects in the avian model. Further research has determined that the environment interacts with, and has lasting impacts on DNA in the brain, represented in the expanding field of “epigenetics.” Relationships between environment, behaviour, and DNA changes (such as methylation and histone modification) have been uncovered, providing further detail to the possible mechanisms of transgenerational behavioural effects (McEwen, 2008). Nätt et al (2009) provided further evidence for transgenerational effects of environment with respect to stress in an avian model. They found that chickens raised in an unpredictable light-dark cycle, which provides feeding stress as chickens tend to not eat in the dark, produced offspring with behaviours reminiscent of their own. The study discovered that the similarities in feeding behaviour between parent and offspring (despite the return to a regular light-dark cycle for offspring, and absence of any parental contact in offspring), were associated with differential gene expression in the brain, and possibly mediated by egg hormone levels. These studies show that stress affects the brain and behaviour, and that these effects can be transferred across generations, potentially via epigenetic mechanisms.

2.3 Spatial Learning
The effects of stress on learning and memory are important measures of behaviour with respect to animal welfare, as higher order cognitive functions such as these are relatively sensitive to environmental stress (Shors, 2006). A large volume of literature regarding spatial learning and birds tends to be focused on either pigeons or food-caching species. The majority of work published on chickens, in relation to spatial learning, is focused on behavioural lateralization as a result of pre-hatching light exposure (Daisley et al, 2009). These studies have indicated that visual lateralization plays a role in navigational strategies in space (left eye attending to global distance between objects, and right eye attending more to local landmark cues) (Tommasi and Vallortigara, 2001). When the importance of global and local landmarks has been investigated in pigeons, it has been found that local cues are of greater significance for searching for food rewards (Cheng et al, 2006). Additionally, Cheng (1989) found that when landmarks were shifted within a test area, the pigeons shifted their search in the direction of the landmark shift, while maintaining the previous distance that the reward was located from the wall. There are a number of different test set-ups that have been used to test spatial learning abilities, with variations affecting reliance on global and local cues, landmark geometric placement, and training periods. Performance in spatial learning tasks has been found to be influenced by extra-maze cues, arena, or maze type, and the socialization and handling experiences of the subjects (Roberts and Veldhuizen, 1985; Colombo and Broadbent, 2000; Gallup and Suarez, 1980). With specific references to testing style (arena / maze type), Colombo and Broadbent (2000) have found that greater success rates and faster task acquisition occurred in an “open field” type spatial task, when compared to a radial arm maze. They suggested that the “open field” concept is more similar to the natural feeding style of pigeons, which may be applicable to the feeding style and spatial learning abilities of chickens as well.
Within the avian taxa, spatial learning is often claimed to be one of the most advanced cognitive functions, as many species rely on food caches, migrate long distances, and require foraging efficiency for survival. The extent of spatial memory capabilities has been found to vary greatly, by such effects as species, sex, hormones, sociality and stress (Healy and Hurly, 1995; Bettis and Jacobs, 2009; Hodgson et al, 2008; Jones et al, 1999; Sandi and Pinelo-Nava, 2007). The precise effects of stress on spatial learning tend to vary, and there is much disagreement regarding the presence of a benefit or deficit resulting from stress. As stress varies greatly in its sources and reactions, and as learning quality varies based on a number of factors (including the experimental definition of “success”) it can be predicted that the interaction will be complex as well (Shors, 2006).

A study by Kleen et al (2006) found that chronic stress, the continued occurrence of a stressor for several days or weeks, has a negative impact on behaviour, impairing not just spatial memory, but motivation as well. Bellani et al (2006) however, tested their subjects prior to a restraint treatment, and found that the effects of stress were dependent on the innate anxiety scores of the individuals. These findings oppose the general statement that stress causes spatial learning deficits, suggesting that stress interacts with particular traits of susceptibility to produce negative effects. Similar predispositions for stress effects were found by Touyarot et al (2004) in a social context. Rats that were classified as “reactive” to novel objects were negatively affected by a chronic social stress treatment (regular exposure to unfamiliar conspecifics) in a spatial task. The control group and unstressed but innately anxious rats, did not display the deficits in performance. In a study on the effects of social isolation, as a social stress, Chida et al (2006) found no effect on spatial learning, but decreased learning ability for an associative task. In complete contradiction to the studies just mentioned, Calandreau et al (2011) produced evidence suggesting that a week of exposure to novel stimuli (often associated with increasing fear responses, and therefore stress) actually improved performance on a spatial memory task compared to unstressed controls. Oomen et al (2010) suggested that while different behaviours may be affected by early stress, the intrinsic stress of the tests contributes to the extent and variety of responses shown. It was found that rats that had been deprived of maternal contact on the third day after birth, later developed impairments in spatial learning ability (a mildly stressful test). Conversely, tests of associative learning and fear conditioning (highly stressful) showed a dramatic increase in performance. This study shows that, in the case of learning and memory, the effects of stress are not strictly positive or negative, but may prepare an individual for future stressful events.

2.4 Aims and Predictions
There were two goals of this experiment. One: to quantify the behavioural effects of early stress (social isolation) on spatial learning in the domestic chicken, and two: to determine if performance on a spatial task could be affected by parental experience (i.e. early stress). We predicted that social isolation would negatively affect the ability to display spatial learning, and that these effects would be heritable.

3 Materials and methods
This studied included two rounds of testing. One with a parental stock, and the other with their offspring. Only the parental birds were exposed to early social isolation. Additionally, due to space and time constraints within the research centre as this project progressed, differences in both handling and testing occurred between the two stocks. Therefore, the parental and offspring birds will be discussed separately with respect to materials, methods, and results.
3.1 Parental testing (P0)

3.1.1 Animals
Two groups of White leghorn chickens were utilized in this experiment. The parental testing batch consisted of 53 birds, 31 males and 22 females, randomly selected for testing from a larger P0 batch (Figure 1). The P0 batch was hatched (24 February 2010) at the animal facility at the University of Linköping, from eggs received from a commercial stock bred for egg production. One day after hatching, all chicks were wing-tagged, vaccinated, and blood tested (for sex confirmation). On the second day post-hatch, the P0 batch was split even into “stressed,” and “control” groups. Both groups were balanced for sex. The stressed group was exposed to the early social isolation stressor. From these stressed and control groups, half were selected for use during testing, and the other half (“naïve”) were maintained for breeding the next generation to avoid dilution of the early stress effects. The end composition of parental testing batch was: 13 stressed and 18 control males; and 11 stressed and 11 control females.

Stress treatment occurred for three weeks. At a randomly selected time point during the light cycle, all stress treatment birds were removed from their home pen, and placed in isolation pens without food or water. Control birds remained in the home pen undisturbed. Isolation treatment times increased from one hour in the first week, to two hours in the second week, and finally to three hours in the third week. All spatial learning tests occurred when the birds were between 16 and 20 weeks old (June / July 2010). Prior to spatial learning tests, individuals from the parental group had participated in various other behavioural tests (open field, social reinstatement, novel object, dominance and associative learning) as a part of a larger project in the research group.

Figure 1. Schematic of group distributions, with respect to treatment and parental stock.
3.1.2 Housing Conditions
All testing was performed at the Wood-Gush chicken facility located at Vreta Högskolan, Östergötland, Sweden. At six weeks of age, all chickens were transported to this facility. The temperature of the pens was approximately 25 degrees Celsius, and was maintained with a 12 hour light-dark cycle. At the research station the animals had access to commercial chicken food and water *ad libitum* (Håkanson and Jensen, 2005; Håkanson et al. 2007). Because of egg production requirements, females were supplemented with food bells of oyster shells (2 per pen). The day before the habituation session, and two days before testing, all birds were blood sampled as a part of other projects. After blood sampling, individuals scheduled for the spatial task were placed in separate pens (separated by sex) located near the testing arena. These temporary pens were 2 m long by 1 m wide and 2 m high. Wire sheets were placed on top of the enclosures, to prevent escape. Floors were covered with a wood-shaving substrate, and birds had *ad libitum* access to perches, food, and water. The chickens had regular exposure to human researchers, and the cleaning staff, reducing the likelihood of fear-of-human stress responses.

3.1.3 Arena
The arena was constructed out of cardboard covered pen segments and was 2 m long by 2 m wide and 2 m high (Figure 2 a). A small hole in the top of the arena allowed for direct visual observation. Held together by plastic Zip-ties, one of the segments could be swung open like a door to gain access to the testing area. The arena was set-up in the food room at Vreta, which could be closed off from the main corridor, making the room dark, and isolating it from noise. Every day before the beginning of the sessions, the grey linoleum floor was mopped, and the arena was set-up. Cardboard cups (7.8 cm high and 7 cm diameter (Figure 2 b)), with plastic lids were taped to the floor at eight locations in the arena. Each cup contained two mealworm food rewards (Figure 2 c). A single 60 w light bulb was suspended above the centre of the arena, 150 cm from the floor, with a switch outside the arena. Between each subject, the arena was cleaned of droppings and swept to maintain the spatial integrity of the arena. Additional visual and olfactory cues could potentially confound any learning effects.
3.1.4 Test procedure
The first day of the experimental protocol involved a habituation period. The 7-12 birds tested per week were divided based on sex for the habituation period (because over the previous weeks, the mixed sex cage arrangements had resulted in the removal of a number of females from the experimental testing group due to severe pecking). Chickens were food deprived for 18 hours prior to habituation to ensure they were adequately motivated during testing. During the one-hour habituation period, the regular chicken feed was distributed near and on the food cups to encourage exploration and reduce novel object effects. After the first batch was collected (post-habituation), the arena floor was cleaned of any defecation, and any knocked over cups were replaced. More feed was added to the floor if necessary. After the groups completed the habituation period, they were returned to the temporary pens along with the food bells. Testing occurred the next day.

At 08:00 on testing days, the food bell was removed from the pen with the first testing group. The first tests began at 09:00, when the food bell from the second pen was removed.
Therefore, all birds that were tested had been food deprived for at least an hour (with a maximum of three hours). Each test began by placing the subject in the darkened arena, between cups A and F, facing the wall. The door segment of the arena was then closed, putting the arena into complete darkness. The light to the arena was then turned on, the stopwatch started, and observations were made through a hole in the top of the arena. The session was finished after all the worms were consumed from all eight cups, or after ten minutes. Behaviours recorded during the session are listed in Table 1. After completion of the trial, the chicken was returned to the temporary holding pen, which included the food bell. Tests were run each day, with one session per bird per day, for four days.

Table 1. Ethogram of behaviours recorded.

<table>
<thead>
<tr>
<th>Behaviour</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cup completed</td>
<td>Chicken visited the reward cup, removing both mealworms. (it could not be seen if the chicken consumed the worms or not).</td>
</tr>
<tr>
<td>Cup visit</td>
<td>When a chick came within a 10 cm diameter of the outer rim of the reward cup.</td>
</tr>
<tr>
<td>Latency to step</td>
<td>The time between the beginning of a session, with the light going on, and the first foot movement of the chicken.</td>
</tr>
<tr>
<td>Defecation</td>
<td>When a chicken defecated in the arena during a session.</td>
</tr>
</tbody>
</table>

3.1.5 Statistics

Because it could not be determined if birds that did not eat mealworms could not learn, or were not motivated, they were excluded from the primary analysis. All data were tested for normality, with only latency to step being normally distributed when log-transformed. The amount of cups completed, cups visited, and defecations were all tested with Friedman test and Wilcoxon signed rank test for non-parametric data for differences between sessions. Data was analyzed in groups of: all birds, control birds, and stressed birds. Mann-Whitney U-tests were performed for comparisons between stress and controls.

Due to normality, latency to first step was analysed with a univariate repeated measures analysis of variance. Univariate was used because the assumption of spherical variance was met (P = 0.003). Within subject factor was: latency. Between subject factors were: treatment and sex, from which the model was built in a backwards-conditional manner, with interaction terms removed first. A significant sex effect prompted analysis of males and females separately. Sphericity was not met in the male model, so multivariate methods were used, with Huynh-Feldt values reported.

Due to the large amount of birds removed for not eating mealworms, overall distributions of performance level were analyzed with a Chi-squared test for distribution. Performance level groups were: poor – birds that ate no mealworms during testing, sufficient – birds that completed between one and seven cups on all test sessions, and proficient – birds that completed all eight cups in any test session.

All means, standard deviations, and proportions are listed in the Appendix.
3.2 Offspring testing (F1)

3.2.1 Animals
The offspring (F1) were hatched in incubators at the animal facility at the University of Linköping (Kruijt fascility), from eggs produced from the breeding of the stressed and control naïve P0 stock (Figure 1), hence “stressed” and “control” for offspring birds refers to the parental treatment group. For breeding, P0 stressed males were paired with P0 stressed females, and P0 control males were paired with P0 control females. The resulting F1 group composition was 8 stressed males, 11 control males, 11 stressed females, and 16 control females.

From hatching, all birds were handled regularly for recording body weight and tarsus length. On the second day after hatching chicks were wing-tagged with an identification tag, vaccinated against Marek’s disease, and weighed. Birds were randomly assigned to the test group (balanced for sex and treatment) from the F1 group. The birds under went the learning test between nine and 11 weeks of age.

3.2.2 Housing Conditions
Temporary pens in Kruijt were 1 m long, by 1 m wide and 1.8 m high, with wood shavings substrate. Water was provided in water bells (two per pen), and starter crumbs were provided on a 30 cm diameter food tray. At two weeks of age, birds were moved to a larger pen, with two large water bells, two perches, and two large food bells. One hour before testing, birds were collected and moved to temporary pens in the “Chick Lab” that contained wood-shavings, water, and a heat lamp. There was no food in the temporary Chick Lab pens, to enforce food deprivation prior to, and during testing.

3.2.3 Arena
Testing arenas were located in the Chick Lab at the University of Linkoping. Four testing arenas were set up for simultaneous data recording with Ethovision (Noldus). Arenas (110 cm long, 70 cm wide, and 30 cm high) were made of matte brown masonite, and had a removable mesh top to prevent birds escaping, but allowing for clear camera recording. A cardboard cup (two attached cups, 15.6 cm high, 7 cm diameter) was stapled to the floor of the arena in each corner. Suspended above each arena were two lamps, and a small video camera, which was connected to a computer. As spatial learning has been shown to occur more quickly with the presence of local cues, a 10 cm x 10 cm (100 cm³) black paper square was placed on the wall at one end of the arena (Figure 3 a), and a 4 cm x 20 cm black paper rectangle (100 cm³) was placed on the wall at the other end. Two of the arenas had the cue placement reversed compared to the other two (e.g. rectangle placed behind the start spot, instead of in front). Each arena was divided into four equal zones (Figure 3 b) within Ethovision to record the latency to enter each zone, as well as the total duration spent in each zone.
3.2.4 Procedure

Training stage
Training occurred over five sessions in four days. The purpose of the training period was to habituate the birds to the arenas and mealworms, reducing potential novel object and environment effects. A single mealworm was placed in each of the four cups, to build an association between the cups and the food reward. Sessions were five minutes long, and began after a bird was placed in each of the four arenas and the lights were turned on. At the end of each session the lights were turned off and the bird returned to the temporary pen while the remainder of the subjects were trained. Each “block,” a session in which four birds were trained, was balanced for sex and treatment. To prevent the influence of arena effects, it was ensured that a bird was never trained in the same arena twice in a row.

Testing stage
Test birds were randomly selected from those that were trained, to attempt to produce groups balanced for sex and treatment (eight stress males, seven control males, eight stress females, and eight control females). This allowed for each testing block to be nearly balanced for sex and treatment, as well as for arena (Table 2). Because only one mealworm was used during the testing stage (placed in the left or right cup opposite the start point), the set-up was also balanced for worm-cup and local cue.

Table 2. Test arena description

<table>
<thead>
<tr>
<th>Arena</th>
<th>Start point cue</th>
<th>Worm side (Test)</th>
<th>Worm side (Reverse)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 Rectangle</td>
<td>Left</td>
<td>Right</td>
<td></td>
</tr>
<tr>
<td>2 Square</td>
<td>Right</td>
<td>Left</td>
<td></td>
</tr>
<tr>
<td>3 Square</td>
<td>Left</td>
<td>Right</td>
<td></td>
</tr>
<tr>
<td>4 Rectangle</td>
<td>Right</td>
<td>Left</td>
<td></td>
</tr>
</tbody>
</table>
Testing sessions began immediately following the last training session and occurred at least twice every day. The testing stage consisted of five identical sessions, followed by a probe test, recovery session, and delayed recovery session. Each session in the testing stage was one minute in length. The subjects were placed into their respective arenas in the dark and the session began when the lights were turned on. During each session Ethovision tracked each birds movement, recording zone latency and duration. After each session, the experimenter recorded if the worm was gone from the cup. During the probe test, the procedure was identical to the other sessions, with the exception of there being no worm present (zone latency and duration still recorded). The recovery session occurred immediately following the probe test, and served to prevent extinction of the worm-cup, and worm-location association. The delayed recovery test occurred 48 hours following the recovery test, and tested temporal memory fixation.

**Reversal stage**

The reversal stage began immediately following the delayed recovery test of the testing stage (same day). The procedure was identical to that of the testing stage, but with the worm placed in the cup opposite that which it was in during the testing stage (Table 2). As in the testing stage, there were five identical sessions, followed by a probe test, and a recovery test. During each session Ethovision tracked each bird’s movement, recording zone latency and duration. After each session, the experimenter recorded if the worm was gone. During the probe test, the procedure was identical to the other sessions, with the exception of there being no worm present (zone latency and duration still recorded). The recovery session occurred immediately following the probe test.

### 3.2.5 Statistics

Success in the arena (if the worm was eaten or not) was analyzed by binomial regression. The model was fit in a backwards-conditional manner, with treatment, sex, and session as fixed factors.

Latency to enter, and duration of time spent in the worm zone were analyzed by Friedman, Wilcoxon signed-rank, and Mann-Whitney U tests for non-parametric data. All 15 sessions were considered before being split between the testing and reversal stages. Due to non-significance on the Friedman tests, Wilcoxon signed-rank tests were only performed between sessions of particular significance: test session five – probe, test session five – recovery, test session five – test delayed recovery, test recovery – test delayed recovery, reversal session five – reversal probe, and reversal probe – reversal recovery. All means and standard deviations are listed in the Appendix.

### 4 Results

#### 4.1 Parental Chickens (P0)

**4.1.1 Cups completed**

The chickens in the control group increased the number of cups eaten over the sessions (Friedman \( \chi^2_{(18)} = 10.011, P = 0.018 \) , as opposed to the stressed birds, which did not increase (Friedman \( \chi^2_{(12)} = 2.000, P = 0.572 \) (Figure 4 a). Post-hoc Wilcoxon signed-rank tests between individual control bird sessions confirmed that only comparing sessions two and four, and three and four failed to be significant (P > 0.05). For example, control birds increased from 5.56 ± 2.684 cups in session one, to 7.28 ± 1.841 cups in session four. No differences between sessions were found for stressed birds (P > 0.05). Between stress and
controls during session three, control birds completed more cups (7.56 ± 1.423) than stressed birds (5.92 ± 2.746, P = 0.037).

4.1.2 Cups visited
No significance differences were found across session for either control or stress (Friedman $\chi^2_{18} = 0.785$, P = 0.853, Friedman $\chi^2_{12} = 4.096$, P = 0.251, respectively) (Figure 4 b). Post-hoc Wilcoxon Sign-Rank tests within stressed chickens showed differences between sessions two and three (respective mean, SD: 12.50 ± 4.927 cups, 17.08 ± 7.573 cups; P = 0.033). There were no differences between any of the session in controls. Within sessions, only session three was different between control and stress (P = 0.043), with a Mann-Whitney U test.

4.1.3 Performance Level
Within treatment and sex split groups, only the frequency distribution of performance level differed in control females with, 20% poor performers, 10% sufficient performers and 70% proficient performers ($\chi^2_{10} = 6.200$, P = 0.045) (Figure 5). Comparing treatments within sex performance groups showed that controls differed from stress treatment only within proficient males (respective proportions: 55.6 and 16.7%; $\chi^2_{14} = 5.333$, P = 0.021). No other group differences were significant (P > 0.05). Only within proficient stress birds, was there even a trend between males and females (respective proportions: 16.7 and 63.6%; $\chi^2_{10} = 2.778$, P = 0.096).

Figure 4. Comparing control and stressed birds for the number of cups completed (a) and cups visited (b) for each session.

Figure 5. Proportion of chickens from each gender and treatment in each performance level.
4.1.4 Latency to first step
Latency to first step was found to be a significant within subject factor, in a univariate repeated measures analysis of variance ($F_{(3,81)} = 14.401, P < 0.001$) as latency varied across sessions (Figure 6). Sex was found to be a significant between subject factor for latency to step across sessions ($F_{(1,27)} = 4.712, P = 0.039$). However, treatment and sex were not found to interact with latency across sessions ($P > 0.05$). Post-hoc Student’s T-test found no significant difference between treatments within sessions ($P > 0.05$).

![Figure 6. Comparing control and stressed birds for the latency for first step for each session.](image)

### 4.1.5 Defecations
There were no significant differences between sessions within the control, or stress birds in a Friedman test ($P > 0.05$) (Figure 7). When both treatments were combined, there was a trend across sessions ($Friedman \chi^2_{(30)} = 7.687, P = 0.053$), but not when considered alone ($P > 0.05$). The trend indicated a decrease across sessions (e.g. stressed, session one: $1.67 \pm 1.231$ defecations; session four: $0.92 \pm 0.900$ defecations), however, Wilcoxon signed-rank, and Mann-Whitney U tests failed to find any significant differences between treatments or sessions ($P > 0.05$).

![Figure 7. Comparing control and stressed birds for the number defecations for each session.](image)

### 4.1.6 Correlations
The number of cups completed for all birds, was strongly correlated to the number of defecations (Spearman’s rho = -0.632, 30 d.f., $P = 0.000$) and the latency to step (Spearman’s...
rho = -0.752, 30 d.f., P = 0.000). The number of cups completed, was moderately correlated to the number of cup visits (Spearman’s rho = 0.374, 30 d.f., P = 0.042). The number of defecations, was moderately correlated to the number of cup visits (Spearman’s rho = -0.555, 30 d.f., P = 0.001) and the latency to step (Spearman’s rho = -0.504, 30 d.f., P = 0.005).

The number of cups completed for control birds was strongly correlated to the number of defecations (Spearman’s rho = -0.695, 18 d.f., P = 0.001), the number of cup visits (Spearman’s rho = 0.727, 18 d.f., P = 0.001) and the latency to step (Spearman’s rho = -0.734, 18 d.f., P = 0.001). The number of defecations was strongly correlated to the number of cup visits (Spearman’s rho = -0.709, 18 d.f., P = 0.001) and the latency to step (Spearman’s rho = -0.709, 18 d.f., P = 0.001). The number of cup visits was strongly correlated to the latency to step (Spearman’s rho = -0.757 18 d.f., P = 0.000).

The number of cups completed for stressed birds was strongly correlated to the latency to step (Spearman’s rho = -0.881, 12 d.f., P = 0.000).

4.2 Offspring Chickens

4.2.1 Successes

No differences between sex or treatment for success on the task was found, in total, or across the sessions (Figure 8). For females, only a trend for treatment (P = 0.061) was found in the binomial regression, but not session (P = 0.449). No factors were found to be significant in the model of only males.

4.2.2 Latency to Enter Worm Zone

No difference was found across sessions or within treatments for all birds, females, or males (P > 0.05) (Figure 9). No difference was found across sessions in the testing stage or within treatments for all birds, females, or males (P > 0.05). No difference was found across sessions in the reversal stage or within treatments for all birds, females, or males (P > 0.05).
4.2.3 Duration in Worm Zone

The control group was found to vary across sessions ($\chi^2_{(14)} = 25.512, P = 0.030$), while the stress group was not in Friedman tests ($\chi^2_{(16)} = 20.561, P = 0.113$) (Figure 10). When split between the two test stages, the stress group was found to differ across sessions ($\chi^2_{(16)} = 14.287, P = 0.046$), while the control group did not ($\chi^2_{(14)} = 11.464, P = 0.120$). The reversal stage found no differences across the session in either treatment group ($P > 0.05$). Within the sessions, only during the delayed recovery, was there a difference between control and stress (respective means: $15.2 \pm 12.4$ seconds, $7.2 \pm 7.5$ seconds; $P = 0.043$). Wilcoxon signed-rank tests between days of interest yielded no significant results for all birds together, or either treatment group ($P > 0.05$).
5 Discussion

5.1 Effects of Social Isolation on Spatial Learning
Our study shows that social isolation stress during early development hampers spatial learning in adult chickens. Across sessions, stressed birds did not increase the number of cups completed, whereas control birds did. The observed indicators of stress (latency to step and number of defecations) decreased over the sessions for both the stressed and control birds, which suggest that the difference in learning between treatment groups was most likely not due to the stressful testing situation. The increase in cups completed was unlikely to be merely due to increased exploration because the number of cups visited did not change across days in the control birds.

Studies have shown that stress can be beneficial for spatial learning (Calandreau et al, 2010), while others have found it to be extremely detrimental (Touyarot et al, 2004). Stress can exhibit both advantageous, and negative effects on learning depending on the intrinsic and extrinsic context of stress (Oomen et al, 2010). It is difficult to disentangle the occurrence of learning from the effects of the intrinsic stress of the testing, so both should be considered when interpreting the results of learning tests (Shors, 2006). Sandi and Pinelo-Nava (2007) suggest that learning and stress may in-fact be indistinguishable, as all behavioural tests contain intrinsic stressors affecting the performance of the subjects. Therefore, one possible explanation for the difference between stressed and non-stressed birds in our study might be that the stressed chickens were less capable of coping with the stressful nature of the testing itself, involving separation from conspecifics and an unfamiliar environment (despite habituation). Previous studies have indicated that exposure to stress can sensitize the brain to react to future stressful situations (Kuzminski et al, 2010), and cause a shift in cognitive functions away from spatial learning (Oomen et al, 2010). Additionally, different stressor intensities can have different behavioural effects, which also display individual variation (Cordero et al, 1998). Bellani et al (2006) illustrated that only rats which were defined as being anxious early in development, before undergoing physical restraint stress, showed impaired spatial learning later in adulthood. Additionally, one month after the restraint treatment, high-anxiety rats had basal cortisol levels three times higher than their low anxiety counterparts, and twice as high as high-anxiety subjects not exposed to the restraint stressor, indicating that individuals vary in not only their sensitivity to stress, but also in the extent of effects.

In our study, the improvement of learning was independent of the other behaviours recorded (stress related: latency to step and defecation; explorative: cups visited). Harris et al (2009) showed that when the effect of thigmotaxis (a stress-related wall-hugging behaviour) was removed, the effects of the treatment on spatial learning ability disappeared. It should be noted however that the Harris study (2009) investigated positive effect (improvements caused by environmental enrichment). The current study, as well as the Harris study, highlights the difficulty in determining actual success in spatial learning paradigms.

5.2 Transgenerational Effects
The second spatial learning test, performed on the offspring generation, failed to show any effects of the early parental stress treatment most likely due to a failure to record learning with the test paradigm we used. We found no apparent improvements between the offspring of stressed and control birds for success in the test, latency to enter the zone with the food reward, and duration of time spent near the food reward. Additionally, the probe tests (indicators that the worms were found by memory and not simply by visual inspection) failed
to validate the test. While this test was unsuccessful in detecting any transgenerational effects of early stress on spatial learning, the same individuals showed differences in other physiological and behavioural measurements (pers. comm., Vivian Goerlich and Per Jensen, IFM, Linköpings Universitet), supporting a long-lasting treatment effect across generations.

While the specific results of this study provide no evidence of a transgenerational effect of early parental stress treatment on spatial learning performance, further phenotypic measurements on the same individuals suggest differences in behaviour and physiology (pers. comm., Vivian Goerlich and Per Jensen, IFM, Linköpings Universitet). These findings have large implications with respect to the effects of early stress, not just on learning, but on the effects of the parental environment on behaviour. Oomen et al (2010) found that early stress improved associative learning in rats later in life, which is similar to what the parental line displayed: improved associative learning (pers. comm., Vivian Goerlich and Per Jensen, IFM, Linköpings Universitet), despite decreased spatial learning performance. What was not investigated by Oomen et al (2010), was the heritability of the effects of stress. By testing the offspring in the present study, we showed that stress during early development has long lasting, transgenerational effects which, however, manifest only in certain behavioural and physiological aspects.

There are several potential underlying mechanisms for transgenerational effects. Physiological stress might affect neurons and germ line cells through a singular mechanism. Epigenetic controls in the brain, such as DNA methylation, and histone modification are related to behavioural changes, specifically with relation to stress and associative learning (Miller et al, 2008). With that in mind, in addition to the discovery that DNA methylation, specifically, can be maintained in dividing germ line cells (Petronis, 2010), it is a reasonable conclusion that stress may cause a global physiological change in DNA methylation which is heritable. For this to be concluded, neural and germ line tissues samples would need to be compared for methylation levels between stressed and non-stressed birds. Corticosterone, due to its role in the avian HPA-axis, and circulation throughout the body, may be a good biomarker for investigation of the source of methylation changes. In the avian model, hormones have the opportunity to have long lasting influences on embryo development by affecting the yolk hormonal environment. Because yolk hormone levels are determined prior to laying, and the egg is an essentially closed system, the period embryo development provides further opportunity for hormonal effects on cognitive functions. The specific mechanism through which early stress could affect egg hormone deposition is, however, currently unknown (Groothuis and Schwabl, 2008). Further study would also be necessary to determine if the heritable effects of stress occur within a particular sensitive period in development, and if they could be overcome.

Understanding the interaction between stress and behaviour is particularly important for maintaining high standards of animal welfare. Animal husbandry practises frequently expose livestock to stressors, but a deeper understanding of the timing of stress, and its effects can limit the negative impact of such events and improve farming practises. Chickens, in particular are often exposed to stress early in development, which we have shown negatively affects cognitive development and behaviour.

5.3 Conclusion
Prolonged stress during early life has a negative effect on spatial learning in adolescent individuals. The transgenerational effects of parental stress on spatial learning were inconclusive in this study, however other offspring behaviours showed differences due to
parental treatment. The effects of stress may influence behaviours through different mechanisms, some of which may be heritable and regulated by epigenetic mechanisms.

6 Acknowledgements
I would like to thank all of the members of the AVIAN research group, for contributing to a stimulating research environment, and supporting me, and this project. In particular, Per Jensen and Vivian Goerlich provided regular support at all stages of this study, and encouraged me throughout the process. Daniel Nätt, and Magnus Elfwing were also invaluable members of the team, and contributed time, effort and knowledge in the various testing stages.

7 References


### Appendix – Means and standard Deviations

#### Table 1. Mean number of cups completed by parental chickens (P0).

<table>
<thead>
<tr>
<th>Session (mean, SD)</th>
<th>N-value</th>
<th>1</th>
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<th>3</th>
<th>4</th>
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<tbody>
<tr>
<td>Control</td>
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<td>5.56</td>
<td>2.684</td>
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<td>2.111</td>
<td>6.25</td>
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</table>

#### Table 2. Mean number of cups visited by parental chickens (P0).

<table>
<thead>
<tr>
<th>Session (mean, SD)</th>
<th>N-value</th>
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<th>4</th>
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<td>4.003</td>
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</table>

#### Table 3. Distribution of performance level by sex and treatment by parental chickens (P0).

<table>
<thead>
<tr>
<th>Performance Level (%)</th>
<th>Poor</th>
<th>Medium</th>
<th>Good</th>
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<tbody>
<tr>
<td>Control Female</td>
<td>20.0</td>
<td>10.0</td>
<td>70.0</td>
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<tr>
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<td>9.1</td>
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<td>63.6</td>
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<tr>
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<td>41.7</td>
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#### Table 4. Mean latency to first peck (mm:ss) by parental chickens (P0).

<table>
<thead>
<tr>
<th>Session (mean, SD)</th>
<th>N-value</th>
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<th>2</th>
<th>3</th>
<th>4</th>
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<tbody>
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<td>02:42</td>
<td>00:22</td>
<td>00:28</td>
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#### Table 5. Mean number of defecations by parental chickens (P0).

<table>
<thead>
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<tbody>
<tr>
<td>Control</td>
<td>18</td>
<td>1.78</td>
<td>1.263</td>
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<td>Stress</td>
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<td>1.67</td>
<td>1.231</td>
<td>1.42</td>
<td>1.832</td>
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#### Table 6. Mean amount of successes across session in offspring chickens (F1).

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<tr>
<th>Females</th>
<th>Males</th>
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<tbody>
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<td>N-value</td>
<td>Mean, SD</td>
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<tr>
<td>Stress</td>
<td>8</td>
</tr>
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</table>

#### Table 7. Mean latency to enter the Worm Zone (seconds) during Testing sessions by offspring chickens (F1).

<table>
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<th>Sessions (Mean, SD)</th>
<th>1</th>
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<th>3</th>
<th>4</th>
<th>5</th>
<th>P</th>
<th>R</th>
<th>DR</th>
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<tbody>
<tr>
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<td>17.5,</td>
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<td>24.0,</td>
</tr>
<tr>
<td>Stress</td>
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<td>15.5</td>
<td>23.9</td>
<td>21.0</td>
<td>23.8</td>
<td>20.6</td>
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<td>15.5</td>
</tr>
<tr>
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<td>27.2,</td>
<td>30.6,</td>
<td>30.2,</td>
<td>29.6,</td>
<td>32.9,</td>
<td>16.7,</td>
<td>22.6,</td>
</tr>
<tr>
<td>Stress</td>
<td>21.0</td>
<td>24.8</td>
<td>24.2</td>
<td>19.3</td>
<td>26.0</td>
<td>24.5</td>
<td>21.9</td>
<td>23.9</td>
</tr>
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</table>
Table 8. Mean latency to enter the Worm Zone (seconds) during Reversal sessions by offspring chickens (F1).

<table>
<thead>
<tr>
<th>Sessions</th>
<th>Mean, SD</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>P</th>
<th>R</th>
</tr>
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<tbody>
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<td>30.3, 20.8</td>
<td>27.8, 24.4</td>
<td>22.5, 17.5</td>
<td>29.4, 19.5</td>
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</table>

Table 9. Mean duration of time spent in the Worm Zone (seconds) during Testing sessions by offspring chickens (F1).

<table>
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<th>Mean, SD</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
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<th>R</th>
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<tbody>
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<td>13.9, 9.7</td>
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Table 10. Mean duration of time spent in the Worm Zone (seconds) during Reversal sessions by offspring chickens (F1).

<table>
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<tr>
<th>Sessions</th>
<th>Mean, SD</th>
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<th>R</th>
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<td>15.7, 10.3</td>
<td>11.6, 11.9</td>
<td>16.3, 12.2</td>
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<tr>
<td>Stress</td>
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<td>15.0, 11.5</td>
<td>16.8, 15.2</td>
<td>9.7, 10.3</td>
<td>11.7, 11.4</td>
<td>8.2, 8.5</td>
<td>14.1, 15.2</td>
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</table>
The effects of early stress on life-time strategies of behaviour and coping in chickens (*Gallus gallus*)

Stress is often an important consideration for animal welfare. A number of factors can contribute to stress in domestic animals, most notably those used in food production. We investigated the effects and heritability of stress in domestic chickens (*Gallus gallus*). Using a spatial learning paradigm, we tested an early social isolation-stressed group and their offspring against unstressed controls, to determine if this cognitive function was negatively affected by stress. In the parental generation, we found that across sessions control birds improved in performance, indicating a learning trend. Stressed birds showed no difference across sessions, indicating a lack of learning. No effects of the parental treatment were found in the offspring of stress and control birds. Social isolation stress was found to affect spatial memory learning, however, we did not find evidence that the parental stress influenced the spatial abilities of the next generation despite changes in other behaviours.