Examensarbete

Tonic immobility and effects of early stress on chickens (*Gallus gallus*)

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Tonic immobility and effects of early stress on chickens (*Gallus gallus*)

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**Abstract**
Tonic immobility, TI, is an innate fear response in many vertebrate animals, induced by brief physical restraint. It is a widespread theory that chickens (*Gallus gallus*) reared under stressful conditions react stronger in tonic immobility tests, i.e. stay still and lay still for a longer period of time, than individuals reared under more stress-free conditions. In our study we attempted to see if stressful conditions early in life (temporary isolation from the flock on a daily basis) had any effect on how the individuals handled the stressful and fear-evoking experience of tonic immobility tests. A total of 77 chickens of the HyLine strain of White Leghorn were used. Three sets of tests were performed; first at the hatchery facility at Linköpings universitet; second after a stressful experience – the moving to the Wood-Gush facility at Vreta jordbruksgymnasium; and a random sample with about half of the birds the third time, also at Vreta. The results were inconclusive, but pointed more in the direction of the early-in-life stress having no effect on TI tests rather than the other way around.

**Keyword**
Tonic immobility, restraint, fear, chickens, early stress, Gallus gallus
Contents
1 Abstract 1
2 Introduction 1
3 Materials and methods 2
   3.1 Animals 2
   3.2 Facilities 2
      3.2.1 Kruijt facility at Linköping university 2
      3.2.2 Wood-Gush facility at Vreta jordbrukskynnasium 3
3.3 Tonic immobility test 3
   3.3.1 Procedure and recordings 3
      3.3.1.1 Day 1 – At 47-49 days of age 3
      3.3.1.2 Day 2 – At 56-58 days of age 3
      3.3.1.3 Day 3 – At 62-63 days of age 3
   3.4 Statistical analysis 4
4 Results 4
   4.1 Induction 4
   4.2 Time until first head movement 5
   4.3 Time until righting 7
5 Discussion 9
   5.1 Induction 9
   5.2 Time until first head movement 10
   5.3 Time until righting 10
6 Conclusions 11
7 References 11
1 Abstract
Tonic immobility, TI, is an innate fear response in many vertebrate animals, induced by brief physical restraint. It is a widespread theory that chickens (Gallus gallus) reared under stressful conditions react stronger in tonic immobility tests, i.e. stay still and lay still for a longer period of time, than individuals reared under more stress-free conditions. In our study we attempted to see if stressful conditions early in life (temporary isolation from the flock on a daily basis) had any effect on how the individuals handled the stressful and fear-evoking experience of tonic immobility tests. A total of 77 chickens of the HyLine strain of White Leghorn were used. Three sets of tests were performed; first at the hatchery facility at Linköpings universitet; second after a stressful experience – the moving to the Wood-Gush facility at Vreta jordbruksgymnasium; and a random sample with about half of the birds the third time, also at Vreta. The results were inconclusive, but pointed more in the direction of the early-in-life stress having no effect on TI tests rather than the other way around.

2 Introduction
When animals are at the very beginning of their lives, the developing brain is experience-sensitive, and experience-dependent. Intervening with the natural environment early in life in humans and primates – e.g. by social isolation – has been shown to have long-term consequences regarding both emotional functioning and social behaviour (Champoux et al. 1989, Chugani et al. 2001). In the wild, rodents that have pups are forced to leave the nest for certain periods of time – minutes to hours – to find food for themselves. In 1957, S. Levine re-modeled this maternal care pattern into the laboratory by early handling, which consisted of isolating pups from the mother and the cage in individual compartments for 15 minutes at a time (Levine, 1957). Animals that were handled as infants showed changes in the functionality of the hypothalamic-pituitary-adrenal (HPA) axis (Levine, 1957) in a way that the adult individual later had an increased ability to respond, cope, and adapt to novel or stressful stimuli (Meaney et al. 1991). Such individuals also showed faster peaks in the release of the stress hormones called glucocorticoids and faster returns to basal levels; interpreted as an adaptive change since high levels of stress hormones circulating in the body over a longer period of time can result in neurotoxicity (Lupien et al. 1998). Over the years, many studies have shown the negative effects of stress (e.g. Plotsky and Meaney 1993; cited by Cirulli et al. 2010). Tonic immobility is an innate fear response, a behaviour induced by a brief physical restraint of some kind, depending on species. It occurs in many vertebrate species including birds, lizards, pigs and rabbits (Valance et al. 2008). In piglets, the tonic immobility can be induced by placing the animal on its back, either in a cradle or on a flat surface, and put a light pressure on its chest and/or hindlegs (Forkman et al. 2007). In poultry and quail the procedure to induce tonic immobility is much the same: the bird is placed on its back in a cradle and is then kept there by gently putting pressure on its chest (and on its head too, sometimes). This method is the most common one used today as it has turned out to be the most consistent and reliable one, with the least number of inductions needed to induce tonic immobility (Jones and Faure, 1981). Chickens, among other animals, elicit this “death feigning” as a response to being faced with a predator. It means that they will play dead to be able to escape when (if) the predator relaxes its concentration. In this case the simulated predator is the experimenter handling the chicken. Fear is an increasingly important factor to account for in the animal production today, because it may lower the animals’ productivity. It has been shown that stress-related behavior can be inherited in both chickens (Janczak et al. 2006, Nätt et al. 2009) and other animals as well (Kapoor and Matthews 2005). The reason why we conducted our experiments with tonic immobility, TI, on our chickens is because it is so widely used and
accepted as a measurement of an individual’s stress level, and level of fear (see also Forkman et al. 2007).

Our main objective during this study has been to examine whether chickens that were reared under more stressful conditions under their first weeks post-hatch would react stronger on a tonic immobility test, i.e. be easier to induce in such a test, and stay in the immobile state for a longer period of time compared with conspecifics reared more stress-free.

The main hypothesis for this study is that stressful conditions early in an individual’s life affect how it handles stress later in life.

3 Materials and methods

3.1 Animals

All in all there were 137 chickens (Gallus gallus) of the White Leghorn HyLine strain in the flock. 80 of these chickens were signed up for the tonic immobility tests and the remaining 57 were so called “naive birds” and were not to be tested at all. However, two TI test chickens had to be put down before the tests began. One chicken fainted during a simple blood sampling, and was therefore excluded from the TI tests, due to the suggested risk that they could be harmful to the chicken. It was still housed with its conspecifics. Hence, 77 chickens – 32 females and 45 males – underwent the tests.

37 out of the 77 TI test chickens had been reared under stressful conditions, i.e. during their first weeks in life they had been isolated from the rest of the flock in a cardboard box, whereas the remaining 40 were reared stress-free. To ensure that all 78 birds had experienced the same amount of handling, the non-stressed chickens were also caught and isolated from the rest of the flock recurrently – however, in small groups instead of alone. The chickens hatched on Feb 24th, 2010. Four days post-hatch, the post-natal stressing treatment was started. On a daily basis one week forward from Feb 28th the 37 chickens were isolated, one at a time, from the rest of the group for one hour. During this time the chicken had no access to food or water, and the temperature was lowered – however, the isolation from the conspecifics is considered to be the major stressor here. The second week the chickens were isolated for two hours daily; and on the third week for three hours.

3.2 Facilities

3.2.1 Kruijt facility at Linköping university

Before the tests started the flock had been divided into two groups, with the 77 TI test chickens in one pen – approximately 3x1.5 m – and the 57 naive chickens in another. Both groups had free access to food and water, and the pens’ floors were covered with wood shavings. When the TI tests were to begin, the pen with the 77 test chickens had been divided into two parts, with approximately half of them on each side. This was done to coordinate the TI tests with other tests. The chickens were 47-49 days old when the tests were performed at the university. One experimenter performed the tests in the laboratory room at the facility, and the other in the main room, in an attempt to minimise the birds’ effect on each other. The lights in the ceiling in the main room and in the laboratory were turned off to try and reduce unnecessary stress levels in the birds. The only light source in the laboratory came from a desktop lamp turned away from the chicken; in the main room a certain amount of daylight was let in through the partially covered windows.
3.2.2 Wood-Gush facility at Vreta jordbruksgymnasium
At an age of 55 days, on the 20th of April, the chickens were placed in previously mentioned cardboard boxes (15 individuals per box) and driven to Vreta in two cars. The flock’s pen of approximately 3x3 m was divided into two parts, where the naive birds were kept on one half and the test birds on the other. Both groups had free access to food and water and the floor was covered with wood-shavings. One experimenter performed the tests in the laboratory room, and the other in the supplies room. The conditions resembled the ones at the university: daylight through shaded windows was the only light source in the laboratory, and a desktop lamp provided the light in the supplies room. The chickens were 56-63 days old when the tests were performed at Vreta.
Between Day 2 and Day 3 a perch system with three levels was installed in the pen to enrich the chickens’ environment. It measured approximately 2.5x1 m.

3.3 Tonic immobility test

3.3.1 Procedure and recordings

3.3.1.1 Day 1 – At 47-49 days of age
One and the same experimenter entered the bird cage and randomly picked two chickens to perform tests on. The lights in the animal room were dimmed upon entering and were turned up again when leaving the room. The chickens’ ID-tags were checked to ensure that no naive bird was accidentally tested. The two chickens were then placed separately in 576x346x407 mm cardboard boxes (Biltema 2010) to calm down from the possibly stressful experience of being removed from the flock. After 3 min the chicken was picked up and placed on its back in a wooden V-shaped cradle (22 cm long with an approximate angle of 90°). The bottom of the cradle was 2 cm above the table’s surface, and was kept there with a light pressure on its sternum for 10 s before release. When releasing the chicken the experimenter slowly backed one step away from the table. If the chicken jumped within 5 s after release it was said not to have entered tonic immobility, and if the chicken had not entered tonic immobility after three attempts the test was terminated. During the whole test, the experimenter stood silent and as immobile as possible until the chicken righted itself or until 10 min had elapsed. After the test the chicken was colour-ringed and let back into the pen synchronously with the other experimenter’s chicken, to reduce the number of disturbant situations for the birds. The number of induction attempts, time until first head movement and time until righting were the measured variables (Forkman et al. 2007). Times were taken with Rucanor stopwatches.

3.3.1.2 Day 2 – At 56-58 days of age
At Vreta, there were two differences in the procedure conditions. First, the lights in the birds’ pen room were dimmed during the whole days when tests were performed, except during lunch. Second, once two chickens had been tested, they were put back into the half of the pen where the naive birds were. This was a simplifying procedure to avoid that the experimenter would repeatedly enter the cage and accidentally catch chickens that had already been tested.

All 77 chickens underwent the test once on Day 1 and once on Day 2.

3.3.1.3 Day 3 – At 62-63 days of age
The second time at Vreta, one week after they had been moved there, 40 chickens were randomly picked and tested again to detect any possible habituation effects. These tests were performed under two consecutive days; this test occasion is called ‘Day 3’ below.
3.4 Statistical analysis
In Statistica 9, one-way ANOVAs were used to determine whether the observer had had any effect on the number of inductions needed; time until first head movement and time until righting, on the different days respectively. Repeated measures ANOVAs were used to compare the different days with each other on the one hand, and whether treatment and sex combined had affected the test results, on the other. General linear model calculations were used to determine whether the time elapsed between the chickens being moved to Vreta, and the day for their first test there (Day 2) affected the results; and to test the random sample (Day 3) against treatment and sex. In all three analysis models a significance level of p<0.05 was used.

4 Results
One significant difference (F(1, 38) = 5.64; P = 0.022) was found comparing the two experimenters, and that was for time until first head movement on Day 3. This means that the chickens that were tested by one experimenter stayed still in the TI cradle for a longer period of time than did the chickens tested by the other experimenter.

4.1 Induction
On Day 1 there was a trend (Figure 1; P<0.1) on the number of inductions needed compared between treatments; control (c) and stress (s) respectively in the figure. This trend suggested that chickens reared under stressful conditions needed more inductions to enter tonic immobility. The trend did not apply for the two latter days, though (P>0.2).

![Box plot diagram on the number of inductions needed to induce tonic immobility on Day 1, for control (c) birds and stressed (s) birds respectively. (F(1, 75) = 2.806; P = 0.098).](image_url)

There was a statistically significant difference (Figure 2; P<0.001) between the Days when comparing the number of inductions needed overall, where Day 3 was clearly separated from the prior two. Treatment and sex were not taken into account.
Figure 2. Graph showing within effect differences, as means, on the number of inductions needed to induce tonic immobility on Day 1-3 (Induk. 1-3). The graph was made from a repeated measures ANOVA ($F_{(2.72)} = 12.353; P = 0.00002$). Vertical bars denote 0.95 confidence intervals.

4.2 Time until first head movement
Results on time until first head movement showed a trend on Day 3, suggesting a difference depending on treatment (Figure 3; $P<0.1$). Chickens reared under stress-free conditions stayed still for a longer period of time than did the ones reared under stressful conditions. This trend was not seen on the two other Days ($P>0.1$).
Figure 3. Box plot diagram on time until first head movement on Day 3 ($F_{(1. 38)} = 3.861; P = 0.057$).

Moreover, a trend was also found (Figure 4; $P<0.1$) plotting the time until first head movement against sex on Day 2. Here, this suggested that male chickens laid still in the cradle for a longer period of time than did female chickens.

Figure 4. Box plot diagram on time until first head movement on Day 2, based on sex ($F_{(1. 75)} = 2.814; P = 0.098$).

When all three Days were included at the same time and the statistics were based on treatment as well as on sex, the results in Figure 5 (below) were obtained. There was a slight increase in
the time until first head movement for the female chickens that had been reared under stressful conditions, while a distinct decrease in that time was noted for the male stressed chickens – both compared with their non-stressed conspecifics respectively.

![Graph on time until first head movement Day 1-3, set against treatment and sex. From repeated measures ANOVA results ($F_{(1.36)} = 6.831; P = 0.013$). Vertical bars denote 0.95 confidence intervals.](image)

**Figure 5.** Graph on time until first head movement Day 1-3, set against treatment and sex. From repeated measures ANOVA results ($F_{(1.36)} = 6.831; P = 0.013$). Vertical bars denote 0.95 confidence intervals.

### 4.3 Time until righting

On time until righting, no significant results were obtained for any single calculation, though the box plots with the highest significance on treatment and sex respectively are presented below (Figures 6 and 7).
Figure 6. Box plot diagram on time until righting on Day 2, based on treatment ($F_{(1.75)} = 0.230; P = 0.633$).

Figure 7. Box plot diagram on time until righting on Day 3, based on sex ($F_{(1.38)} = 0.864; P = 0.359$).

When Days 1-3 were all included at once, a significant difference was found between them ($P<0.05$) and the graph below was obtained (Figure 8). Treatment and sex differences were not taken into account here.
Figure 8. Graph showing within effect differences on righting times, as means, between Day 1-3. The graph is from a repeated measures ANOVA ($F_{(2, 72)} = 4.872; P = 0.010$). Vertical bars denote 0.95 confidence intervals.

5 Discussion

Overall, the results from this study discard the hypothesis that stressed chickens would handle the stressful situation differently. One can mainly advocate for either of two popular possible explanations concerning how early stress affects the individuals. Either you could say that if the animals have been exposed to stressors early in life, e.g. our case of social isolation, they would have somewhat ‘experience’ of it and therefore handle similar situations better when facing them later. Results of this kind have been obtained before (Jones and Faure, 1981). In this 1981 study, regular handling of the birds resulted in reduced responses in TI tests later conducted. However, as also discussed in the mentioned study, it seems more like the chickens were habituated to the experimenters and the specific situations rather than a general depression of fearfulness. On the contrary, you could say that since these animals were exposed to stressors early in life they might associate handling with a negative (stressing) experience and therefore be more stressed later in life as well, when faced with similar situations.

At least two studies (Gentle et al. 1989, Nash et al. 1976b) found that, following the very onset of the tonic immobility, heart rate, muscle tone and sympathetic nervous activity (the one elevated under stress) were all lowered during the whole test – heart rate even reached its lowest point right before the chickens rightened themselves. If there is a critical point in heart rate which would serve as a switch for the chickens to terminate the immobility, then one could suppose that more stressed chickens would have a higher heart rate at the induction of the tonic immobility and perhaps take longer time to reach that low.

5.1 Induction

Higher susceptibility for tonic immobility, i.e. lower numbers of inductions needed as well as longer periods of time until first head movement and righting have been interpreted as signs of a higher level of stress (Jones, 1992). With this in mind, the results on inductions are interesting yet maybe not too favorable for our hypothesis. The fact that the stressed chickens on Day 1 needed more attempts to induce TI do indicate that they would be less stressed than
the control group, according to most studies’ theories (e.g. Campo et al. 2005). Perhaps it could be explained by that the stressed birds had experience from isolation before and therefore didn’t get as stressed by this treatment. Since this trend was nowhere near as clear on Day 2 or 3 albeit similar (with the control birds slightly lower than the stressed ones; data not shown), it actually could just be coincidence on that first Day. Both the control birds and the stressor-reared ones had been handled by experimenters equally much, so that parameter is accounted for. The general pattern with the number of inductions being significantly higher on Day 3 regardless of sex and treatment points possibly towards a habituation effect. This was the third time in barely three weeks that these birds underwent the test. It might be that this frequent handling and exposure to the tonic immobility test habituated the birds for just this situation. This explanation is in line with prior suggestions to explain this pattern (Nash et al. 1976a, Jones and Faure 1981b). Nash et al. showed that, quote; “repeated elicitation of immobility, and not just handling, was responsible for reduced response durations after multiple exposures to manual restraint”. It could also be an effect of all the chickens living together – as they did between Day 2 and Day 3. A recent study done on rats showed just that – the rats that had been reared communally, and not the handled rats, showed differences in brain structures and -functions, and a more adaptive coping style to stressful stimuli (Cirulli et al. 2010).

5.2 Time until first head movement
The difference on Day 2 between the sexes (Figures 4 and 5) is intriguing even if the p-value isn’t very low. This difference, that female chickens had shorter times until first head movement has been observed before (Jones and Faure 1981a). Jones and Faure found no obvious explanation for this apparent difference and neither have I, but it might be as they suggested – that, quote: “...the female allows herself more time to ‘explore’ the environment for potential dangers before righting and flight”. If so, the reason behind it is to me still unknown. Maybe the reason behind such an explanation would involve different hormones and their levels circulating in the body.

Figure 3 showed a trend that the stressed birds had lower times until first head movement than did the control birds. (This pattern was seen on Day 1 and Day 2 as well but not that clear at all (data not shown). However, it was increasingly clearer with the highest p-value on Day 1.) Maybe this too has to do with the nerve system’s development and stressed chickens being able to cope with stressors presented to them later, better than their conspecifics – as implied on inductions above.

One thing that is important to keep in mind here is that this is the one variable where we had a significant difference between the experimenters. The chickens were picked randomly but it later turned out that one experimenter had tested nearly twice as many stressed birds as control ones; and this one experimenter also had significantly lower times until time to first head movement. The difference showed in Figure 3 could therefore be an unfortunate result of the human error.

5.3 Time until righting
Starting with the most significant figure, Figure 8 shows a trend between the Days with effects similar to those seen in Figure 2. The fact that the righting times were lower on Day 3 than on the two prior ones might fall in line with the habituation effect and/or the effect of social enrichment (all chickens living together in the pen) mentioned earlier. The fact that the perch system was installed between Day 2 and Day 3 might have had a positive effect on the chickens’ nerve system development as it serves as a social enrichment compared to an empty pen.
Figure 6 with its graph on righting times on Day 2 suggests that there is no difference between the treatments on this variable. This is a bit of setback as we had hoped to see a clear difference on righting times, especially between Day 1 and Day 2. We discussed in terms of that the stressed chickens possibly would stay longer in tonic immobility on Day 2, after the stressful experience of being moved to a novel environment (Mitchell and Kettlewell 1994, Graml et al. 2008).

Figure 7, showing sex differences on time until righting on Day 3, is somewhat in line with Figure 4 and with previous research (Jones and Faure 1981a). The same kind of tendency seemed to apply to our experiment, as it did in theirs – namely that male chickens not only had a higher latency until first head movement but also had a shorter time between time until first head movement and time until righting (data not shown). Even if this indeed is the fact, it is to my knowledge unknown why it is like that.

6 Conclusions
In this study, the stress that the chickens were exposed to early in life did not generate any conclusive results on how these individuals would react to stressful treatment. Our results therefore leave the hypothesis – stressful conditions in an individual’s life affect how it handles stress later in life – unanswered. It is possible that stressors early in animals’ lives really do affect how they handle stressful situations later in life, but to answer that I think even more standardised test conditions are required, in combination perhaps with a larger population. A more complex test setting might also help; maybe by checking EKG and/or hormone levels at the same time would help providing clearer results. The results from this study do not recurrently support the theory that more stressed chickens uniformly will stay longer in tonic immobility than non-stressed conspecifics.

7 References

Cirulli, F; Berry, A; Bonsignore, L.T; Capone, F; D’Andrea, I. et al. (2010). Early life influences on emotional reactivity: Evidence that social enrichment has greater effects than handling on anxiety-like behaviours, neuroendocrine responses to stress and central BDNF levels. Neurosci. and Biobeh. Reviews 34, 808-820.


Valance, D; Després, G; Richard, S; Constantin, P; Mignon-Grasteau, S. et al. (2008). Changes in Heart Rate Variability during a tonic immobility test in quail. Physiology & Behaviour. 93, 512-520.