Leukocyte activity as a proxy to measure stress in birds using house sparrows (*Passer domesticus*) as a model species

Diploma Thesis

University of Veterinary Medicine Vienna

submitted by
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Plagiarism Declaration

I hereby declare that the assignment on the topic mentioned above

1. Is the result of my own independent work,

2. Makes use of no other sources or materials other than those referenced, and that quotations and paraphrases obtained from the work of others are indicated as such.

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Date                                           Signature
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1. Introduction

1.1 Definition of Stress

The phenomenon of stress is an important topic and field of research in almost all disciplines as it affects animals and humans in equal measure and in multiple ways. It causes the organism to respond with a combination of complex mechanisms that help to cope with environmental change and challenges.

To explain and define the term itself has been a continuous effort since the start of stress research in the early 20th century. As the first, Selye formed the term and defined it as “a nonspecific response of the body to any demand” (Selye 1976). Furthermore, he defined the term “stressor” as “all endogenous or exogenous agents” triggering such a response (Selye 1976). The response is primarily the release of glucocorticoid hormones and catecholamines that affect different metabolic systems in order to respond to a challenging agent. Consequently, in regard to those response mechanisms, stress was also defined as “any stimulus that will provoke simultaneous increases in plasma glucocorticoid concentration and in glucocorticoid secretion rates” (Yates 1967). For glucocorticoid release was (and still is) seen as the main response mechanism overall. However, as there are many different regulatory systems involved, it is important not to disregard other players of the stress response.

When focusing on the purpose of the reaction, the complex response mechanisms aim to an adaptation of the organism to those stimuli (Ewbank 1973) and to maintain homeostasis (Cannon 1932). Yet, the organism needs to be continuously in a homeostatic state and all metabolic systems work to achieve that goal lifelong and apart from the classical stress response. Therefore, highlighting homeostasis when defining the aims of stress responses might not live up to the actual purpose (Koolhaas et al. 2011). Koolhaas proposes that stress should rather be limited to situations that are beyond “the natural regulatory capacity of an organism”.

In consideration of the ongoing discussion and despite intensive research, the concept of stress remains problematic and disputable. Stress responses are multifaceted and possess a
high complexity on different levels. Thus, it is pivotal to enhance and illuminate well-established as well as new methods of measuring stress in order to advance towards a more in this thesis we will refer to stress and stressor as follows: Capture and physical restraint during handling that induce a complex stress response at different functional levels (hormonal, cellular, immunological) in order to maintain homeostasis.

1.2. Physiology of Stress Response

Selye did not only provide the word “stress” itself and its first definition, but also discovered the importance of corticosteroid hormones and their role as major players in the stress response. He named the series of reactions to a stressor the “general adaptation syndrome” (Selye 1976). This covers three stages: First comes the “alarm reaction” which can show itself initially as a fight or flight reaction. Second, there is the “stage of resistance” where the organism tries to adapt to an ongoing stressful stimulus, and third, the “stage of exhaustion” focusing on chronic stress that reduces the ability to adapt and cope with the stressor (Selye 1936). Over the years this concept has been extended by new data and research (Selye 1976; Charmandari et al. 2005).

Figure 1: The general adaptation syndrome

The two main neuro-hormonal systems that participate in regulating the response to a stressor are the hypothalamic–pituitary–adrenal axis (HPA) and sympathetic-nervous system (SNS). The SNS is a fast (within seconds) reacting regulatory system that induces a “fight or flight response” by releasing epinephrine (adrenalin) and nor-epinephrine from the adrenal medullary. To be able to react to an acute situation, digestive function is inhibited, periphery vessels constrict with the consequence of increasing heart rate and blood pressure, ventilation and perfusion of the lungs is enhanced and energy is provided by release of glucose (Romero & Butler 2007).

The HPA axis is the neuroendocrine system regulating the release of corticosteroid hormones (in humans and most mammals cortison, in birds and rodents mainly corticosterone). If a stressing stimulus reaches the central nervous system (CNS), a specific area in the brain, the hippocampus, is activated first and induces the cascade of the axis (Jacobson & Sapolsky 1991). Additionally, further parts of the brain are involved such as the amygdala and the brainstem (Herman et al. 1996).

The hypothalamus, more specifically the lateral paraventricular nucleus (PVN), is being signalled by a stressing stimulus to synthesize and release corticotrophin-releasing factor (CRF) and arginine vasopressin (AVP) (Engelmann et al. 2004). These hormones on the other hand stimulate the anterior pituitary gland to secret adrenocorticotrophin (ACTH) from corticotrophic cells.

However, the axis is not only regulated by the mentioned neurotransmitters, but also by other components of cells and immune system, especially cytokines such as interleukin-1 and

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Figure 2: HPA axis

-6 (IL-1, -6) or tumor necrosis factor-α (Besedovsky & Del Rey 2000). These elements affect the corticotrophic cells and therefore directly influence the release of corticosteroids. ACTH is only one of the hormones that alongside cytokines activate the adrenal cortex and induce the production of corticosteroids from adrenocortical cells (Marx et al. 1998).

Corticosteroids bind to mineralocorticoid receptors (MR) with high affinity and glucocorticoid receptors (GR) with lesser affinity in the hippocampus as well as other cells around the body (de Kloet et al. 1990).

The cascade is regulated by different positive and negative feedback mechanisms. The increase of the plasma corticosteroid level for example induces a negative feedback on the hypothalamus as well as the anterior pituitary and thus, directly limits the secretion (Keller-Wood & Dallman 1984; Siegel 1980).

However, the HPA is not a linear regulatory system, but rather a complex process that is influenced by numerous mediator substances of different physiological systems. Consequently, it is appropriate to state that it requires different methods of measuring the stress response in order to understand this complexity as detailed as possible and in its entirety.

When it comes to avian species, the corticosterone (CORT) secretion is generally regulated by the same mechanisms although the avian pituitary seems to be more independent from the influence of the hypothalamus (Siegel 1980). In case of hypophysectomy, baseline CORT is almost unaffected whereas in mammals, it is much lower (Baylé & Assemacher 1967).

Corticosteroids affect the organism in several ways to ensure metabolic balance and to adapt to a stressor (Romero & Butler 2007). One effect is the increase of blood glucose in order to provide energy. This is achieved by reducing the resorption of glucose from the blood and by mobilising glucose reserves. Glycogen is transformed into glucose and new glucose is formed through gluconeogenesis. These mechanisms ensure a stable supply of glucose for parts of the system that are particularly required in that situation (e.g. muscles) (Romero 2004).

Another effect is the inhibition or reduction of systems that are not primarily needed in coping with a stressor such as growth (Sapolsky 1992) and reproduction (Wingfield, John & Romero
2001) as well as the immune system. The immunosuppressive effect (as a consequence of chronic stress) results in blocking different elements of the innate and adaptive immune system such as T-, B cells, macrophages and other phagocytes, lymphocytes, cytokines or the major histocompatibility complex (Spencer Robert L. et al. 2001). However, some studies indicate that short-term increase of plasma glucocorticoid concentration might even enhance the immune function and suggests that glucocorticoids influence the immune system in a context dependent manner (Dhabhar 2009).

1.3. Leukocytes and the Respiratory Burst

As previously mentioned, stress may be suppressive to parts of the immune system. This also includes the first line in defence when eliminating invading pathogens: the leukocytes. Not only is the number of circulating leukocytes reduced after a highly stressful event (Maxwell 1993), but also their ability to react to pathogens (McLaren et al. 2003). Leukocytes are non-specific phagocytes, primarily heterophils in birds (Maxwell & Robertson 1998). Their main task is the disposal of bacteria by phagocytosis.

This is a complex mechanism and is achieved by a number of mechanisms. The pH is increased to 3.5 – 4.0 to create an intracellular bactericidal environment. Enzymes such as lysozyme are produced that destruct cell walls of some gram-positive bacteria. Competitors are released that constrain the bacterial metabolism (Murphy et al. 2009). However, the most important factors are bactericidal products such as antimicrobial peptides, toxic nitrogen

Figure 3: Respiratory burst in mammals

http://www2.phys.rush.edu/TomD/RespBurstNew.JPG

8
oxides and reactive oxygen species (ROS). The reaction to bacterial agents results in an increase of oxygen uptake by granulocytes and the production and release of ROS. This is called the respiratory burst. ROS are not only bactericidal and an important part of phagocytosis, but also pathogenic to own body cells (Råberg et al. 1998).

The production of ROS starts by transforming O2 into superoxide O2(−) through NADPH-oxidase. O2(−) is then transformed into hydrogen peroxide H2O2 which is an effective ROS itself. H2O2 is then transformed to hypochlorous acid HOCl and other ROS by the enzyme myeloperoxidase (MP) (Murphy et al. 2009).

However, in contrast to mammals birds do not possess MP and thus, do not produce HOCl. Studies indicate that the respiratory burst is nonetheless a powerful tool of eliminating microbial pathogens in avian species (He et al. 2007).

1.4. Leukocyte Coping Capacity

The ability of leukocytes to respond to a secondary challenge after being exposed to a stressor is called leukocyte coping capacity (LCC) (McLaren et al. 2003).

The receptors of leukocytes are susceptible to factors such as cytokines that accumulate in the blood in reaction to stress. Leukocytes that were exposed to these factors show a decreased LCC (Mian et al. 2005). When stimulated with an artificial trigger substance (e.g. phorbol 12-myristate 13-acetate [PMA]), the production of ROS through the respiratory burst is induced. The emerging ROS can then be quantitatively measured in whole blood by chemiluminescence. As a baseline reference poses an unstimulated sample that is mixed with a buffer substance (McLaren et al. 2003; Shelton-Rayner et al. 2012).

The majority of studies on mammals used luminol as lumigenic agent. In birds, however, the intensity of the chemiluminescent response is much higher when using lucigenin (Makarskaya et al. 2011).

The LCC test is a relatively new method that is a rapid and adequate way to measure the current physiological status of an organism in vitro after a short-term stress impact (McLaren et al. 2003; Gelling et al. 2009; Shelton-Rayner et al. 2011).
1.5. Objective of the study

Many fields of biological research such as ecology, physiology and animal husbandry involve capturing and handling of animals. This inflicts stress and affects them in multiple ways. It is therefore pivotal to study adequate tools of assessing and measuring stress on animals in a quantitative manner to be able to optimize capture and handling procedures.

Studying stress in avian species poses a special challenge as the volume of blood samples is very limited. The leukocyte coping capacity (LCC) technique is a possible approach to that problem. It requires a small amount of blood and is practical under field conditions as it can be measured right away without considerable post-processing (see Methods).

There have been several studies that have already measured ROS production in avian species using chemiluminescence (Conlon et al. 1991; Desmidt et al. 1996; Papp & Smits 2007). However, only a few have put it into the context of stress (Mujahid et al. 2007).

In this study, LCC was measured to test its practicability as a technique to quantitatively and objectively assess the short-term stress response in avian species. LCC was measured alongside corticosterone (CORT) to verify a possible correlation between those parameters.

Furthermore, stress responses in birds are subject to seasonal variations (Romero 2002). CORT responses are significantly lower in winter than in spring. To assess whether this also applies to LCC, animals were sampled in winter and in spring to detect possible variation in both parameters. The study was designed to improve the understanding of stress responses in avian species regarding capture and handling in different seasons. The knowledge about the LCC technique will be further expanded and compared to classical measurements of stress hormones. Illuminating stress responses provides essential information concerning animal welfare and is useful to reduce stress in future studies with direct human-animal interaction.

The following hypotheses are questioned in this study: The experiment will add the novel technique of LCC to the existing research on house sparrows. Stress levels of CORT will be low right after capture and reach their peak after 30 minutes (de Kloet et al. 2005). We assume that LCC levels will be higher after the first sampling compared to the second after being kept in a resting bag for 30 minutes. The stress response of birds in winter will be significantly lower than in spring regarding both stress parameters (Romero 2002).
2. Methods

2.1. Study species

For our experiment, 40 house sparrows (*Passer domesticus*) (19 males, 21 females) were used as a study species. They pose an excellent model to answer the stated hypotheses in various ways. These passerine birds live in most regions around Europe and all around the globe (Summers-Smith, 1988). Their main food consists of grains and weeds, but they also commonly eat insects and other foods (Anderson 2006). The house sparrow is a human commensal and well adapted to rural as well as urban surroundings. Their husbandry is easy and uncomplicated and therefore suitable for the experiment. Furthermore, there are several other studies that have used this species before and confirm some of our hypotheses by measuring stress responses through hormonal analysis (Romero et al. 2006). Even though it has to be taken into account that the above studies where conducted with free-living birds in contrast to our captive study, our results can still be put in an adequate context and comparisons can be made.

2.2. Study area

The birds were kept in outdoor aviaries at the Konrad Lorentz Institute of Ethology in Vienna. Each aviary accommodated a small colony of maximum 15 birds of the same gender. A few aviaries was inhabited by birds of both gender. They were housed under natural light conditions and ambient temperature. Aviaries were equipped with small fir trees, plants and branches. Sufficient feeding plateaus and wooden perches were available. The birds were fed once a day in the morning with canary seed mix and foxtail millets. Once a week they were given a piece of apple and additional special bird food that is rich in protein. Water was available at all times and the aviaries were cleaned once a week. Feeding routine was always adapted to the current season and specific requirements.

All animals were held under these conditions prior to as well as during the experiments.
2.3. Study design

The birds were captured in the weeks from 04/01 until 18/01/2016 and 14/03 until 25/03/2016 at 9:30 (+/- 2 minutes) using dip nets. From each bird two blood samples were taken via puncture of the Vena ulnaris using a small sterile cannula (2x 70 µl = 140 µl in total; two heparinised 20 µl capillaries for LCC analysis and two heparinised 50 µl capillaries for hormonal analysis). The first blood sample was taken right after capture, the second after 30 minutes staying in a cotton bag.

The team usually consisted of eight persons. Three persons were in charge of entering one aviary and catching four birds at a time. This approach was necessary in order to capture the birds as fast as possible to ensure that blood samples were taken within 3 minutes. This is the maximum time period in which an appropriate corticosterone (CORT) baseline can still be determined (Romero & Romero, 2002; Romero & Reed, 2005).

Two experienced persons took the blood sample of two birds each. In parallel, one assistant per team was in charge of recording the procedure by taking the times, putting the birds in the predetermined bags and ensuring a smooth flow in general. Timers were started when the team approached the aviaries. An overrun of 30 seconds was tolerated. The time when entering the aviary, taking the blood sample (start and end), time in the cotton bag and also the time of taking the second blood sample were recorded (in seconds).

The capillary for hormonal analysis was filled first (as this is the most time-dependent sample) following the capillary for LCC analysis. Punctuation wounds were pressed on for 20 seconds at least to reduce further blood loss. Capillaries for LCC analysis were directly handed to the eighth and last person who transferred the samples into previously prepared tubes filled with Lucigenin solution. Samples for hormonal analysis were transferred into tubes, immediately centrifuged for 10 minutes at 3000 U/min and stored frozen at -80 °C for further analysis later on.

After taking the ring numbers of each individual, the sampled birds were transferred into a cotton bag for 30 minutes (+/- 2 minutes).

The cotton bags and the LCC samples were taken into a quiet laboratory close by with as little disturbance as possible. The bags were hung onto hooks on the wall. Inside the room,
necessary communication was reduced to a minimum with lowered voice. After 30 minutes, the second round of sampling was conducted in the laboratory. The order of the first round was kept and the time for the second bleeding started when the birds were taken out of the bags.

Subsequent to the second bleeding, weight, the tarsal length in cm as well as fat- and muscle-scores were taken by the same experienced person to ensure a consistent rating. Birds were released into two aviaries again, separated by gender in preparation of the next round of the experiment in spring.

2.4. LCC analysis

Before each sampling period, chemicals were prepared freshly and stored at the required temperature in a freezer. Each day, the solutions were mixed anew in labelled wide mouth bottles. Phosphate-buffered saline (PBS) was produced with distilled water. Lucigenin and phorbol 12-myristate 13-acetate (PMA) were unfrozen by ambient temperature.

39 ml PBS was then mixed with 1 ml of $10^{-4}$ mol l$^{-1}$ Lucigenin in a brown glass bottle as it is photosensitive. Afterwards, 9,9 ml PBS was added 0,1 ml of $10^{-5}$ mol l$^{-1}$ PMA in another bottle (Shelton-Rayner et al. 2012).

For each day of the experiment, 16 non-reflecting tubes (Lumivial, EG & G Berthold, Germany) were marked with the ID of the individuals to be sampled that day (baseline sample with PBS and challenge sample with PMA; for each run). The baseline tube was then filled with 180 µl Lucigenin solution. 20 µl whole blood would later on be added during the sampling so that half of the mixture (100 µl in total) could be pipetted into the challenge tube. The tubes were wrapped in aluminium foil to ensure that a minimum of light reaches the photosensitive solution.
during sample taking.
After adding the blood sample to the previously prepared baseline tubes with 180 µl Lucigenin solution, half of the mixture (100µl) was pipetted into the correspondent challenge tube. 10 µl PBS was added to the tube that served as a baseline reference, 10µl PMA to the challenge tube (Shelton-Rayner et al. 2012).

The tubes were stored in a preheated metal bead bath at 40 °C covered with a polystyrene lid during the whole experiment and between measurements.
For each tube chemiluminescence was measured in relative light units (RLU) every 10 min in two portable chemiluminometers (Junior LB 9509, EG & G Berthold, Germany) for a total of 80 minutes. Measuring time was 30 seconds for each tube.
After putting the blood samples into the tubes, after adding the chemicals and before each measuring, the tubes were shaken gently. All data were recorded in a protocol.

2.5. Hormonal analysis

Plasma corticosterone (CORT) was measured in June 2016 in the laboratory of Dr. V. Canoine at the Department of Behavioural Biology, University of Vienna.
It was measured after a Dichlormethan-extraction using a commercial available CORT 125I radioimmunoassay kit (catalogue no. 07-120102; MP Biomedicals, Solon, OH, USA). The protocol of the company was followed with small modifications as described by Washburn et al. (2002). The volume of all reagents was divided by two; the dilution of the samples was 1:50 instead of 1:200 and the standard curve was extended of 2 points resulting 8 standard points to increase the sensitivity. Even though this protocol had been validated for different bird species, we repeated the validation for our species and could confirm the parallelisms.
Reagents were brought to room temperature prior to use. Mouse serum was diluted 1:50 with steroid diluent. 0,15 ml steroid diluent was added to non-specific-binding tubes 1 and 2 (NSB) and 0.05 ml to 100%-binding tubes 3 and 4 (0 tubes). 0.05 ml corticosterone calibrators were added to tubes 5 thru 16 and 0,05 ml of reconstituted controls plus (1:50) mouse serum to
tubes 17 to end of assay. After that, 0,1 ml corticosterone 125I was added to all tubes. Additionally, 0,1 ml anti-corticosterone was added to tube 3 to end (not to NSB 1 and 2). The assay tubes were then vortex mixed and incubated at room temperature (22-25°C) for 2 hours. Afterwards, 0,25 ml precipitant solution was added to all tubes and again vortex mixed thoroughly. After centrifuging the assay tubes at 2300-2500 rpm for 15 minutes, the supernatant was aspirated. Now the precipitate was counted in a gamma counter.

2.6. Statistical analysis

Statistical analysis was performed by using the statistical program R, Version 3.2.2 (https://www.r-project.org).

To analyse whether there is a significant difference of average means between the different measurements, one-way and two-way analysis of variance (ANOVA) were used. The significance level was set at p < 0,05. Normal distribution was assessed by displaying the residuals of the linear model in a histogram. If residuals were not normally distributed, variables were transformed using a box-cox transformation.

Homogeneity of variance was assessed with Levene's test.

Regarding LCC, the integral of the LCC curve that describes the progression of the curve during the 80 minutes measuring period was calculated (Area under the curve [LCCauc]). Rather then focussing on single values alone, it enables to view the leukocyte stress response as a whole.

LCC peaks, LCCauc, CORT concentrations, the gradient of both LCCauc and CORT between first and second round of sampling (DeltaAUC, -CORT) and a calculated index of LCCauc and CORT (IndLCC, -CORT) functioned as dependent variables.

The formula for calculating the indices is as follows:

\[
\frac{(CORT_{30} - CORT_{0})}{(CORT_{30} + CORT_{0})}
\]

\[
\frac{(LCCauc_{2} - LCCauc_{1})}{(LCCauc_{2} + LCCauc_{1})}
\]

15
By calculating those indices, a corrected version of the stress response between first and second sampling was tried to be achieved.

The dependent variables were season, sex and the different time sequences in seconds.

**LCCauc2 ~ Sex*Season**

> Model1=lm(LCCauc2 ~ Sex*Season)
> hist(residuals(Model1),col="blue")
> boxcox(lm(LCCauc2 ~ Sex*Season))

> LCCauc2_trans=bcPower(LCCauc2,0.25)
> Model2=lm(LCCauc2_trans ~ Sex*Season)

> anova(Model2)

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<th>Mean Sq</th>
<th>F value</th>
<th>Pr(&gt;F)</th>
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<td>780.02</td>
<td>4.0136</td>
<td>0.0487 *</td>
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<tr>
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</table>

Signif. codes:  0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1

> leveneTest(Model2)

Levene's Test for Homogeneity of Variance (center = median)

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**Figure 5**: Example of statistical analysis (here: LCCauc2 was compared with Sex and Season in a two-way ANOVA)
We tested whether the CORT concentration of the first sample taken right after capture (CORT0 = baseline) was significantly lower compared to the second sample taken after 30 minutes (CORT30). Furthermore, as CORT0 is expected to be highly influenced by the time that is required for taking the blood sample (Romero & Romero 2002), the elapsed time from capture was also considered in the analysis.

We also tested whether CORT30 correlates with LCCauc1 or LCCpeak1 as the CORT concentration of the stress impact usually takes 30 minutes to reach its peak in the plasma due to the slow inertia of the HPA axis.

To test the efficiency of resting bags in reducing the stress of birds while being handled, it was analysed whether LCCauc2/LCCpeak2 was significantly higher (whereas high numbers indicate a greater immune competency) than LCCauc1/LCCpeak1.

As one of the main predictions, we clarified whether birds sampled in winter had indeed significantly lower stress responses compared to birds sampled in spring regarding all stress parameters recorded in this study. A possible interaction between the independent variables season and sex was tested by using a two-way ANOVA.
3. Results

The statistical analyses did not show any significant effect of season on the LCC variables (LCCpeak1+2, LCCauc1+2, IndLCC, DeltaLCC). Thus, in the present study the parameter LCC could not detect any seasonal variation of the stress response. However, both LCCauc1 ($F = 5.1; P = 0.027$) and LCCauc2 ($F = 4.0; P = 0.049$) varied between sexes (Figure 6 and 7).

*Figure 6: Variation of LCCauc1 regarding sex and season*
Figure 7: Variation of LCCauc2 regarding sex and season

CORT variables, on the other hand, varied seasonally. Baseline CORT levels (CORT0) (\(F = 25.7; P = 2.753 \times 10^{-6}\)) as well as CORT levels after 30 minutes (CORT30) (\(F = 15.7; P = 0.000\)) showed significant seasonal variations. The same applies for IndCORT (\(F = 7.8; P = 0.007\)) and DeltaCORT (\(F = 6.6; P = 0.012\)). Average means of CORT0, CORT30 and DeltaCORT were lower in winter and higher in spring. Only IndCORT was lower in spring and higher in winter (Figure 8).
Baseline corticosterone levels (CORT0) were significantly affected by the timespan needed for the blood sampling ($F = 6.6; P = 0.012$). The scatter plot (Figure 7) shows that CORT0 increases with time. There are currently two different time periods that are being discussed as the maximum limit of determining approximated baseline CORT values: 2 minutes (120 seconds) and 3 minutes (180 seconds). Both have been marked in the diagram. Most of the data points can be found in the time period of 3 minutes and possess a small variance in their value. After 3 minutes CORT levels undergo a sharp increase. However, differences can be found regarding sex and season (see Figure 7).

There was no correlation between CORT- and LCC values. Furthermore, the LCC response (LCCauc1~2, LCCauc2~1) of the birds did not improve after staying in a resting bag.

Figure 8: Seasonal variation of CORT variables
Table 1: Arithmetic mean of CORT parameters

<table>
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<tr>
<th>Season</th>
<th>Sex</th>
<th>CORT0 (ng/ml)</th>
<th>CORT30 (ng/ml)</th>
<th>DeltaCORT</th>
<th>IndCORT</th>
</tr>
</thead>
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<tr>
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<td>15,17</td>
<td>76,13</td>
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</tbody>
</table>

Figure 9: Baseline corticosterone (CORT0) in relation to time period of sample taking (in seconds)
4. Discussion

The birds of the study showed a consistent response to the event of capture and handling. Both parameters, LCC as well as CORT concentrations, increased. This has already been documented for many species regarding CORT (Wingfield & Romero 2001) and some species regarding LCC (McLaren et al. 2003; Gelling et al. 2009). Consequently, the LCC technique can be well added to existing research methods on avian species to further illuminate the complex respond mechanisms of stress.

The CORT response follows a seasonal pattern. Baseline CORT as well as CORT after 30 minutes were higher in spring than in winter. The seasonal variation was greater in males. This conforms to previous studies with free-living house sparrows (Romero et al. 2006). However, in the latter study baseline CORT remained relatively stable throughout the seasons whereas in ours seasonal variation could also be detected in baseline concentrations. It remains to be discussed to which extent results of studies with free-living birds can be compared to ones with captive individuals.

Captive-reared birds possess a lower immune responsiveness than free-living birds (Ewenson et al. 2001). It was also shown that CORT responses of free-living house sparrows (being human commensals) were not dependent on habitat conditions (Romero et al. 2006). Therefore, it is possible to draw conclusions when comparing results of groups in different habitats.

To determine baseline values of CORT concentrations that can also be validated as a reference for an unstressed status is a difficult task. Previous studies have shown that CORT concentrations hardly rise within the first 3 minutes after a stressful event (Wingfield et al. 1982). However, there is some evidence that CORT rises already after 2 minutes (Romero & Reed 2005). In this study, blood samples were taken within 3 minutes and also here CORT begun to rise after 2 minutes. However, the increase was not as sharp as for the outliers that were collected after 3 minutes. This confirms previous results. Concentrations of samples taken within 3 minutes can therefore be validated as baseline values with a high likelihood.
LCC results did not show any seasonal variation in contrast to previous studies that found that immune activity underlies seasonal fluctuation and is increased in the winter months (Nelson 2004). The size of immunological organs and the quantity of cells related to the immune system differ seasonally (Nelson et al. 2002). Nevertheless, the LCC technique quantifies the ability of leukocytes to react to a pathogen (respiratory burst) instead of defining their numbers. Therefore, it can be said that the quantity of cells may differ seasonally, but the ability to produce a respiratory burst does not.

Additionally, it has to be considered that data on LCC are still very scarce as this is a rather new technique. There is also a high individual as well as interspecific variability. Further studies may provide more information about that issue.

No seasonal variation in the LCC results was detectable, however, we found differences between sexes for the variables LCCauc1 and LCCauc2. Male house sparrows showed a higher leukocyte activity in winter as well as in spring compared to females. This is in line with studies on other bird species that show a generally lower immune response of males in comparison with females (Zuk 1996; Hasselquist et al. 1999). Male birds possess higher levels of the sex steroid testosterone which is supposed to have a suppressive effect on the immune system (Folstad & Karter 1992).

Several studies measuring the stress response in birds through CORT have unfolded that male birds respond with a higher increase in CORT during breeding in spring (Astheimer et al. 1995; Wingfield et al. 1992; Romero et al. 2006). However, other studies on house sparrows (Breuner & Orchinik 2001) and other species (Romero et al. 1998) could not reaffirm these findings. In this studies, CORT did also not show any variation of sex in CORT.

Recently a study on brown bears showed that LCC does not correlate with classical stress parameters such as heart rate or glucocorticoid concentrations (Esteruelas et al. 2016). The results of our experiment are in agreement with those findings. CORT parameters did not show any correlations with LCC parameters. This can be explained when considering that both parameters represent different regulatory systems. The HPA axis and the release of glucocorticoids is a lot slower than the activation of leukocytes (Sapolsky et al. 2000). It takes
up to 30 minutes for CORT levels to reach their peak (de Kloet et al. 2005). Moreover, glucocorticoids directly influence the distribution of leukocytes. Where catecholamines are responsible for an immediate increase of leukocyte concentration (McCarthy & Dale 1988), glucocorticoids decrease their circulating number afterwards (Pedersen et al. 1994). Thus, when it comes to the stress response, the secretion of glucocorticoids can be rather seen as a compensatory system that keeps essential physiological parameters in a certain range to regain homeostasis.

However, some sources indicate that rather than suppressing immune function glucocorticoids promote the infiltration and distribution of leukocytes to target tissues (Dhabhar & McEwen 1999). Consequently, this naturally results in a decrease of circulating leukocytes.

In summary, measuring the leukocyte coping capacity is a valuable method of assessing the immune response in birds to an acute short-term stressor such as capture and handling. It is especially practicable under difficult field conditions. To which extent this method can be taken to validate the overall effect of environmental and climate conditions such as season is still questionable and hast to be further clarified.

Measuring the glucocorticoid concentration is still an important and well-established instrument of stress research in birds as well as other species. In this study, it detected a seasonal variation of the stress response in house sparrows. It has shown that the stress response is influenced by a number of internal and external conditions. Even though glucocorticoids affect the immune system and immune response, CORT concentration does not directly correlate with LCC levels.

It has to be noted that the distance between both experimental periods was rather short because of organisational limitations. Results might have been more distinct if the birds had been sampled at the peak of the breeding season.

Overall, the results of this study show that the regulation of the stress response indeed possesses a high complexity on different levels which has yet to be fully determined by further research.
5. References


6. Appendix

Capture and Handling Protocol

**Project:**
Using Leukocyte activity as a proxy to measure stress in passerine bird (*Passer domesticus*)

**Date:** __________

**Season:** Winter □ Spring □

**Number of assistants:** ______

**ID:** ______

**Ring Number:** ______

**Aviary Number:** ______

**Sex:** female □ male □

**Resting bag?** yes □ no □

**Time entering aviary:** __________

**Time catching bird:** __________

**Time blood sampling:** __________

**Weight:** ______g

**BMI:** ______

**Comments:**
________________________________________________________

________________________________________________________