Studying the Stress Response across Tissues in House Sparrows; A Dive into the Rest of the Picture through the Lens of DNA damage

An Honors Thesis for the Department of Biology

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Abstract

For several decades, measuring plasma corticosterone levels has been a staple in studying the stress response. However, a consistent endocrine profile of a chronically stressed vertebrate remains elusive. More recently, metrics downstream of corticosteroid release such as red blood cell DNA damage have been studied using model organisms such as house sparrows (*Passer domestricus*). However, the biological relevance of blood DNA damage is unknown and baseline levels of DNA damage across multiple tissue types must first be established to provide points of reference. This project has sought to study those background levels of DNA damage and contextualize measurements in the blood. We assessed the background levels of DNA damage in the blood, abdominal fat, hippocampus, hypothalamus, and the liver in wild-caught house sparrows in the winter and summer to look at seasonal variation in the endocrine profile and applied previously performed and validated chronic stress protocols to captive house sparrows to explore the sensitivity and scope of this metric.

This project of contextualizing DNA damage and identifying an endocrine profile is not complete, but the progress and findings on the technique corroborate the promise of DNA damage as a metric for measuring stress and the overall physiology of the stress response. It is my hope that this work can be continued and that these techniques can further research and our understanding of the stress response.

1. Experiment 1 Introduction

Seasonal variation in the glucocorticoid arm of the stress response has been studied extensively in vertebrates and avian systems for decades (Romero and Wingfield, 2016; Romero, 2002; Wingfield et. al, 1992). Glucocorticoids are steroidal hormones that are generally understood to be upregulated through the stimulation of the hypothalamo-pituitary-adrenal (HPA) axis (reviewed in Sapolsky et. al 2000; Romero and Wingfield, 2016) and comparative endocrinologists have focused on their study in large part due to the wealth of research on their physiological functions and intracellular mechanisms in the biomedical field (Sapolsky et. al, 2000). Seasonal variation in catecholamines, epinephrine and norepinephrine, is comparatively understudied (Romero and Wingfield, 2016). These signal molecules are upregulated by and mediate the effects of the other major arm of the stress response, the sympathetic or "fight-or-flight response." The limitations of the research are likely related to the logistical challenges of measuring baselines catecholamines as they are upregulated almost instantaneously (Romero and Wingfield, 2016). However, measurements of plasma glucocorticoid concentrations have increasingly been deemed to only provide a limited snapshot of the overall endocrine phenotype, or profile, of an organism (Gormally et. al 2019a; Gormally et. al 2019b; Romero et. al, 2015; Sapolsky et. al 2000).

Studying seasonal variation in the stress response and its different mediators allows for the testing of various hypotheses related to stress physiology. Prominent hypotheses include, but are not limited to, the behavioral hypothesis and the preparative hypothesis. The behavioral hypothesis proposes that seasonal variation in glucocorticoid titers is dictated by whether or not glucocorticoid-mediated behaviors are advantageous or disadvantageous during a particular life history stage (reviewed in Romero and Wingfield, 2016). Comparatively, the preparative

hypothesis proposes that seasonal variation in glucocorticoid titers has permissive effects that allow for increased responsivity in non-glucocorticoid mediators of stress during periods of predictably higher stress (reviewed in Romero and Wingfield, 2016). To date, findings have varied across species and taxa and no one hypothesis has been able to unequivocally explain the patterns observed. For example, plasma glucocorticoid titers in Eastern Red-spotted showed no apparent relationship to breeding activity despite this being common in other amphibians (Berner et. al, 2013). Similarly, in house sparrows, some metabolic tissues such as subcutaneous fat and the liver show decreased glucocorticoid receptor density during late breeding life history stages, but no metabolic tissues ever shared a peak in receptor density (Lattin and Romero, 2015). This suggests that the stress response is not solely governed by energetic demands and that responses happen in tissue-specific manners (Lattin and Romero, 2015).

More recently other metrics downstream of glucocorticoid and catecholamine release such as DNA damage have been used to measure physiological responses to acute (Flint et. al, 2007; Malandtakis et. al, 2016) and chronic stress (Gormally et. al, 2019b). DNA damage is directly impacted by catecholamines via independent and synergistic pathways (Flint et. al 2007; Hara et. al 2011). β_2-adrenoreceptor stimulation via catecholamines can increase production of damage-inducing reactive oxygen species and activate Mdm2 through the PI3K/AKT pathway to inhibit and degrade p53, a powerful cellular check on DNA damage and mediator of repair and apoptosis (Hara et. al, 2013). Measuring accumulated DNA damage can therefore be a way of integrating the activity and effects of these hormones as opposed to just measuring their titers. Moreover, the same avian systems commonly used in studying the stress response, such as house sparrows, *Passer domesticus*, have nucleated erythrocytes (Fischer et. al 2018; Lattin et. al, 2013; Love et. al, 2017). The relatively non-invasive acquisition of blood samples can therefore

allow for a variety of stress physiology metrics, including bacteria killing capacity, plasma glucocorticoid titers, and blood DNA damage to be measured from a single sample (Gormally et. al, 2019b). However, the biological relevance of DNA damage in erythrocytes, which are generally not nucleated in other taxa, and what it can tell us about DNA damage elsewhere in the body is not fully understood.

This protocol set out to measure DNA damage across a variety of tissues involved in the stress-response: abdominal fat (sites of metabolic activity and energy mobilization), blood (delivery of glucocorticoids and catecholamines), hippocampus and hypothalamus (brain structures associated with integration of stressors and both stimulation and regulation of HPA axis), and the liver (primary metabolic target of glucocorticoids and catecholamines) in wild and captive house sparrows during the summer and winter (Reviewed in Romero and Wingfield, 2016). This is first time that DNA damage levels in free-living house sparrows will be compared across seasons and tissue types. These data will provide baseline profiles and allow for contextualization and validation of previous and future work looking at DNA damage in avian red blood cells. Based on previous work on free-living house sparrows in Massachusetts demonstrating increased total corticosterone titers in the winter, we expect background DNA damage to be higher be higher in birds during the winter (Romero et. al, 2006).

1. Experiment 2 Introduction

Chronic stress, or the pathology associated with the long-term secretion of mediators of the stress response, has been studied extensively from a biomedical perspective (Sapolsky et. al, 2000), but the variation across taxa and individuals within them suggests a uniform phenotype does not exist (Dickens et. al, 2013; Romero and Gormally et. al, 2019b). The stress response is generally studied through its two major response pathways, the hypothalamo-pituitary-adrenal (HPA) axis (Berner et. al, 2013; Fischer et. al, 2018; Romero, 2002) and the sympathetic fight-or-flight response (Fisher et. al, 2018; Romero & Wingfield, 2016; Sapolsky et. al 2000). Research has specifically focused on the mediators of these pathways, glucocorticoids and catecholamines, and the physiological metrics associated with them.

Briefly, glucocorticoids are steroidal hormones that upregulate the transcription of several effector proteins associated with basal function in an organism. In general, an acute stressor is understood to stimulate the HPA axis and transcriptionally upregulate effectors associated with immediate survival and downregulate effectors not considered immediately essential, such as reproduction (Sapolsky et. al, 2000). Epinephrine and norepinephrine, or catecholamines, are mediators of the sympathetic nervous system that travel to target tissues and stimulate immediate physiological changes associated with survival and increased metabolism (reviewed in Romero and Wingfield, 2016). Traditionally, plasma glucocorticoid titers have been a primary means of measuring an animal's stress response, but what these snapshots leave out are hormone binding and activity, and ultimately some of the physiological repercussions of their fluctuations (Dickens and Romero, 2013; Lattin and Romero, 2014).

The upregulation of the HPA and sympathetic arms of the stress response has been linked to increases in accumulated DNA damage via catecholamines and β_2-adrenoreceptors stimulation

(Hara et. al, 2011; Hara et. al, 2013). The activation of Gs-PKA and β-arrestin-1 signaling pathways via chronic stimulation of the adrenergic receptors results in the production of agents of damage, reactive oxygen species, and the degradation of essential moderators of damage, p53 tumor suppressor proteins (Hara et. al, 2011; Hara et. al, 2013). Protocols exploring DNA damage as an indicator of stress alongside other commonly used metrics such as plasma glucocorticoids (Gormally et. al 2019b; Malandrakis et. al, 2016) suggest that this metric may be useful for integrating the physiological effects of stress. Studying DNA damage across tissues involved in different phases of the stress response may therefore further our understanding of the physiological implications of chronic stress throughout the body (Lattin and Romero, 2013; Lattin and Romero, 2014; Lattin and Romero, 2015). Moreover, this approach may reveal interesting correlations in how these tissues are affected and contribute to the needed validation and contextualization of the commonly used metric of DNA damage in blood (Lattin et. al, 2015; Gormally et. al, 2019b; Malandrakis et. al, 2016).

This study explores the effect of a validated chronic stress protocol (Lattin and Romero, 2014; Gormally et. al, 2019b) followed by a period of 2 weeks of recovery (Lattin and Romero, 2014; Gormally et. al, 2019b) on DNA damage in the abdominal fat, blood, hippocampus, hypothalamus, and liver of captive house sparrows. These tissues were chosen because they all play roles in the stress response; abdominal fat and the liver as primary metabolic targets though which energy stores are mobilized (Lattin and Romero, 2013; Romero and Wingfield, 2016), the hippocampus and hypothalamus as regulators of stimulatory signals and key regulators of negative feedback (Lattin and Romero, 2015; Romero and Wingfield, 2016), and blood as the delivery system of these mediators of stress (Gormally et. al, 2019b; Romero and Wingfield, 2016; Sapolsky et. al, 2000). We hypothesize three weeks of chronic stress in captivity will

increase DNA damage in blood tissues (Gormally et. al, 2019b; Malandrakis et. al 2016) but effects on the other tissues will likely be less predictable and tissue specific. It is likely that more metabolically active tissues like the liver and abdominal fat will undergo high exposure to these stress mediators but differences in cell turnover rates may play a role in the presentation of overall damage (Baquedano et. al, 2011; Dollé et. al, 1997; Pellettieri et. al, 2007). Interpreting these presentations is therefore difficult as the case could be made for faster cycling of cells replacing damaged cells more quickly, but also for the faster cycling of cells with damaged DNA and inhibited mechanisms of DNA repair resulting in a greater accumulation of DNA damage (Hara et. al, 2011; Pelletieri et. al, 2007). A recovery period of two weeks is hypothesized to reduce DNA damage as peak changes in glucocorticoid profiles are observed within two weeks of introduction to a chronic stress protocol (Lattin and Romero, 2014; Gormall et. al, 2019b).

2. Experiment 1 Methods

2.1 Experimental Design

Wild house sparrows were caught using mist nets in two groups, summer and winter, during their respective seasons at the same site in Medford, MA, USA. The summer cohort of wild-caught sparrows consisted of six males and three females (n = 9) caught on July 2, 2019 and the winter cohort of wild-caught sparrows consisted of five males and four females (n = 9) caught on January 13, 2020. Captive house sparrows used for the study were originally caught in Medford, MA, USA using mist nets, but left to acclimate in captivity for at least four weeks such that physiological systems could stabilize to captivity (Fischer, 2018). These birds were housed individually or in male-female pairs in cages (45 cm × 37 cm × 33 cm) on a 12 L:12D light cycle (n = 5, 2M:3F) or 9L:15D light cycle (n = 8, 5M:3F), and were provided water, seed, and grit *ad libitum*. This experiment was approved by the Tufts Institutional Animal Care and Use Committee and was conducted in compliance with the Guidelines for Use of Wild Birds in Research (Gormally et al., 2019a).

2.2 Sacrifice, Sample Collection, & Preparation of Cell Suspensions

Once captured by mist net, birds were brought to an on-site, closed-off procedure room. Blood samples of at least 20 uL were taken from the alar vein within two minutes of capture and stored on ice. Birds were anesthetized and sacrificed by decapitation and the hippocampus, hypothalamus, abdominal fat, and liver were collected, in that order. All tissues were stored on ice in 1.5 mL microcentrifuge tubes containing 1 mL of chilled PBS (Ca^{2+}, Mg^{2+} free, assume all PBS in this protocol is). Prior to their transfer to microcentrifuge tube with PBS, liver samples were cut and left to sit in a weigh boat filled with chilled PBS for ~1 minute to remove unwanted blood. Sample collection was completed between 10 and 20 minutes of capture.

As in other protocols using house sparrows, cell suspensions of blood were prepared by diluting 2 uL of whole blood into 800 uL of chilled PBS followed by two five-fold dilutions (100 uL of suspension into 400 uL of fresh PBS) (Gormally, 2019b). Cell suspensions of tissues other than blood were prepared based on protocols developed in the lab (unpublished). Abdominal fat, hippocampus, hypothalamus, and liver sample were minced in the original 1 mL of PBS they were transferred to, using fine shears. The liver suspensions underwent one five-fold dilution (100 uL of suspension into 400 uL of fresh PBS). Captive birds were sampled in same way, only they were captured from cages.

2.3 Comet Assay

30 μL of each suspension was diluted in 300 μL of warm, low-melting agarose. After tube inversion, 30 μL of sample were plated via pipette such that the gel spread evenly throughout well of the CometSlide purchased from Trevigen (Gaithesburg, MD, USA). Samples were plated in replicate and complete slides were transferred to fridge at 4 °C to solidify gel for 30 minutes. Still in the fridge, the slides were then completely submerged in lysis buffer (10 parts Trevigen buffer to 1 part DMSO) for 60 minutes. The slides were tapped dry and transferred to clean plastic tray and completely submerged in electrophoresis buffer (500 mM sodium acetate, 100 mM Tris base, pH 10) for 30 minutes. A pH of 10 was used as opposed to a higher pH of more alkaline assays after troubleshooting in lab demonstrated less unwinding of DNA at more neutral pH, allowing for reduction of un-analyzable "over-damaged" comets (Gormally et. al, 2019b; Collins et. al 2008). Next, the slides were electrophoresed in fresh electrophoresis buffer for 30 minutes (21v, 6 °C). Following electrophoresis, the slides were soaked in chilled dH20 for two five-minute cycles to neutralize. The slides were then soaked in 70% ethanol for 5 minutes and

heated at 37 °C for 20-40 minutes to dry slides of excess liquid and evaporate gels. Dried slides were stored with desiccant in cool, dark places until ready to be stained and imaged.

This was all done under low light conditions to reduce DNA-damage by UV light (Gormally et al., 2019b). Standardized damaged cells (Catalog #4257-010-NC, R & D Systems, Minneapolis, MN, USA) were plated and run with samples during each comet assay. These standardized cells allow for an estimation of variation between assays and can be used to normalize data if necessary (Gormally et al., 2019b).

2.4 Slide Staining, Microscopy, and Image Processing

Once dry, the slides were stained with SYBR Gold (Molecular Probes, Eugene, OR, USA) for 30 minutes. Excess stain was removed by rinsing slides with chilled dH_2O. Slides were then dried at 37 °C for ~20 minutes and imaged using fluorescent microscope with a green fluorescent protein filter at a 10x objective. The Fiji plug-in OpenComet was used to detect comets in the images taken and abnormal or erroneous detections were manually removed by blinded investigator RE (Gyori et. al, 2014).

2. Experiment 2 Methods

2.1 Experimental Design

25 house sparrows were assigned to three different treatment groups: no treatment (control), chronic stress protocol (CSP), and CSP with a recovery period (CSP + Rec). All house sparrows were caught in Medford, MA, USA, housed individually or in male-female pairs in cages (45 cm × 37 cm × 33 cm), and kept on a short-day light cycle (9L:15D). In order to allow for physiological stabilization to captivity, all house sparrows used had been in captivity for at least four weeks prior to the beginning of their respective treatment (Fischer et. al, 2018). The control group consisted of five males and four females (n = 9**)** and underwent a 21 day period of no treatment prior to sacrifice and sampling. The CSP group (n= 9, 5M:3F) was exposed to a 21day chronic stress protocol as validated by the Romero Lab (Gormally et al., 2019a). The CSP consisted of the administration of four daily stressors, of which three varied in stressor type and time of day administered and one, obligatory fasting, was performed daily at the same time. The variable stressors were chosen randomly and administered in 30-minute increments at random times from the time lights were turned on until one hour after lights were switched off. These stressors included cage rolling (rolling of cart cages sit on), cage tapping (intermittent tapping of object along cage), radio noise, and human voice (researcher reading aloud in room with birds) (Gormally et al., 2019a). Obligatory 18-hour fasts were induced daily by removing food one hour before lights were turned off and returned it two hours after lights were switched on. The CSP birds were sacrificed and sampled immediately after completing CSP. The Rec+CSP group birds were also treated to a CSP but were given fourteen days non-treatment captivity to allow for recovery prior to sacrifice and sampling, as has been done in other recovery experiments (Gormally et al., 2019). This experiment was approved by the Tufts Institutional Animal Care

and Use Committee and was conducted in compliance with the Guidelines for Use of Wild Birds in Research (Gormally et al., 2019).

2.2 Sacrifice, Sample Collection, & Preparation of Cell Suspensions

On the morning of the final day of their respective treatment, birds were caught from cages and sampled. Once captured, birds were brought to a separate, closed-off procedure room. Blood samples of at least 20 uL were taken from the alar vein within two minutes of capture and stored on ice. Birds were anesthetized and sacrificed by decapitation and the hippocampus, hypothalamus, abdominal fat, and liver were collected, in that order. All tissues were stored on ice in 1.5 mL microcentrifuge tubes containing 1 mL of chilled PBS (Ca^{2+}, Mg^{2+} free, assume all PBS in this protocol is). Prior to their transfer to microcentrifuge tube with PBS, liver samples were cut and left to sit in a weigh boat filled with chilled PBS for ~1 minute to remove unwanted blood. Sample collection was completed between 10 and 20 minutes of capture.

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This was all done under low light conditions to reduce DNA-damage by UV light (Gormally et al., 2019b). Standardized, damaged cells (Catalog #4257-010-NC, R & D Systems, Minneapolis, MN, USA) were plated and run with samples during each comet assay. These standardized cells allow for an estimation of variation between assays and can be used to normalize data if necessary (Gormally et al., 2019b).

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taken and abnormal or erroneous detections were manually removed by blinded investigator RE (Gyori et. al, 2014).

*2.5 *Post-Hoc Statistical Analysis of Currently Available Data from Experiments 1 and 2*

Unfortunately, due to a combination of technical issues and limited access to laboratory facilities in the wake of social-distancing measures during the spring of 2020, analysis of data for the two experiments as originally intended has had to be delayed. In place of the original analyses, we analyzed the samples of the wild-caught summer cohort from experiment 1 (n = 9, 6M:3F, from here on referred to as "wild summer") and two cohorts of captive birds. The thirteen total captives consisted of the cohort of captives from the summer in experiment 1 (n = 5, 2M:3F, here on referred to as "summer captives") and those that served as controls in experiment 2 (n = 8, 5M:3F, here on referred to as "fall captives").

2.6 Calculation of Mean Tail Moments and Standardization between Assays

From the various parameters OpenComet provides for each individual cell analyzed, we selected tail moment. This parameter measures the amount of DNA content in the tail, zone to which damaged loops and fragments of DNA migrate to, while taking into account the DNA content of the entire comet and is commonly used to reduce noise with this technique (Olive and Banáth, 2006). Larger tail moment values suggest a greater proportion of the total DNA content exists in "tail" and therefore larger mean tail moments correlate to higher levels of DNA damage.

Wild summer birds and summer captives (n = 5) were run in two assays with minimal variation, but it was observed that birds run in the third assay, fall captives (n = 8), had much lower tail moments. This was attributed to an artificial inflation of total DNA content of all assay three samples. This DNA content inflation was attributed to non-biological reasons associated with inter-assay variation in the staining and imaging of the samples and we normalized the sample of

run three by recalculating tail moments from manipulated total DNA content values. Total DNA content values from all three assays were averaged and divided by the average of samples from assay three, 2.55. All cells of assay three (fall captives) were then standardized by dividing them by this value.

To test for significant differences in mean tail moment values across tissue types within cohorts, the non-parametric, Kruskall-Wallis one-way analysis of variance tests were performed. Spearman's rank correlation coefficients were calculated for all pairwise comparisons of tissue types amongst all birds regardless of cohort (Fig. 2) and then distinguishing by captive or free-living status, such that correlations were calculated for all captive birds (summer and fall captive cohorts, n = 13, Fig. 3) and for all wild birds (summer wild cohort, n=9, Fig. 4). For all statistical tests p values of 0.05 or less were necessary to reject the null hypotheses, and all tests were run using open source RStudio software. Note, some tests had reduced degrees of freedom due to four summer wild individuals having no abdominal fat and the lack of abdominal fat or hypothalamus samples for one fall captive individual.

3. Results

3.1 Comparisons across Tissue Type within Cohorts

There were no significant differences in mean standardized tail moment values across tissue types within the summer wild cohort (Kruskall-Wallis Rank Sum, chi-squared = 3.6494, df = 4, p = 0.46, Fig. 1), the summer captive cohort (Kruskall-Wallis Rank Sum, chi-squared = 4.6671, df = 4, p = 0.32, Fig. 1), or the fall captive cohort (Kruskall-Wallis Rank Sum, chi-squared = 2.5989, df = 4, p = 0.63, Fig. 1). Note, all damage was within range of no damage and low damage standardized, damaged control cells.

3.2 Pairwise Correlations between Tissue Types and by Captivity Status

There were statistically significant, positive correlations of moderate strength between blood and hippocampus (r = 0.4584, S = 834, df = 20, p = 0.0380, Fig. 2), hypothalamus (r = 0.7157, S = 378, df = 19, p = 0.0005, Fig. 2), and liver samples (r = 0.5844, S = 640, df = 20, p = 0.0062, Fig. 2). There was also a statistically significant, positive correlation of moderate strength between abdominal fat and liver samples (r = 0.7147, S = 194, df = 15, p = 0.0025, Fig. 2). Similar patterns arose when looking within only captive individuals. There were statistically significant, positive correlations of moderate strength between blood and hippocampus (r = 0.6538, S = 126, df = 12, p = 0.01832, Fig. 3) and hypothalamus samples (r = 0.7692, S = 66, df = 11, p = 0.0052, Fig. 3) as well as between liver and abdominal fat (r = 0.6643, S = 96, df = 11, p = 0.0221, Fig. 3) and hippocampus samples (r = 0.6318, S = 134, df = 12, p = 0.0237, Fig. 3). Interestingly, there were no statistically significant correlations between any tissues when looking within only wild individuals (no p values < 0.05, Fig. 4).

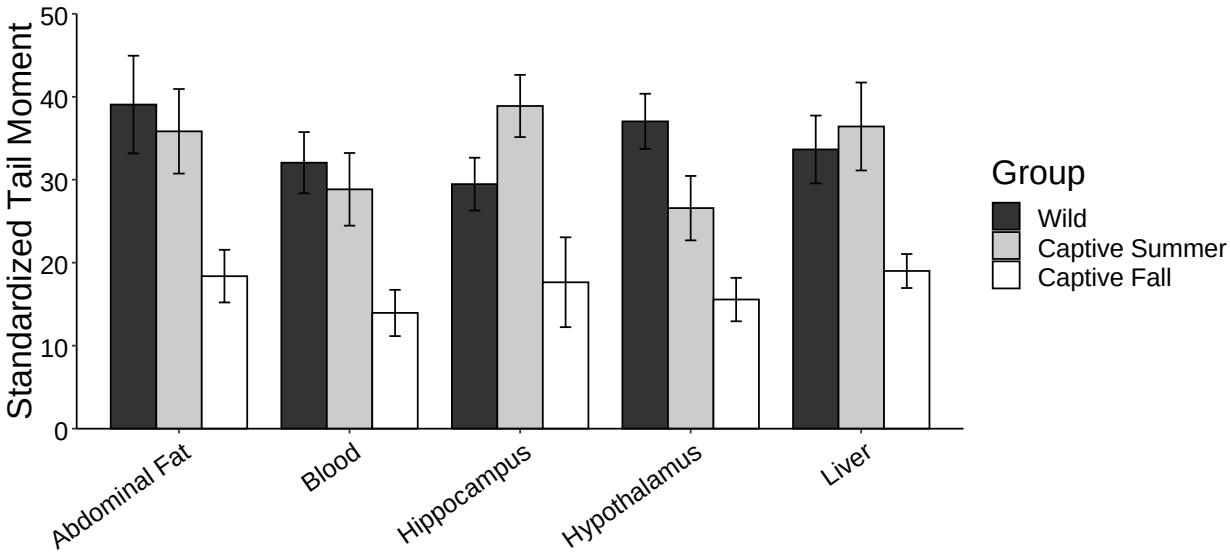

Figure 1. Mean tail moment values across tissue types, within three different cohorts: wild summer, captive summer, and captive fall. Error bars represent mean±SEM.

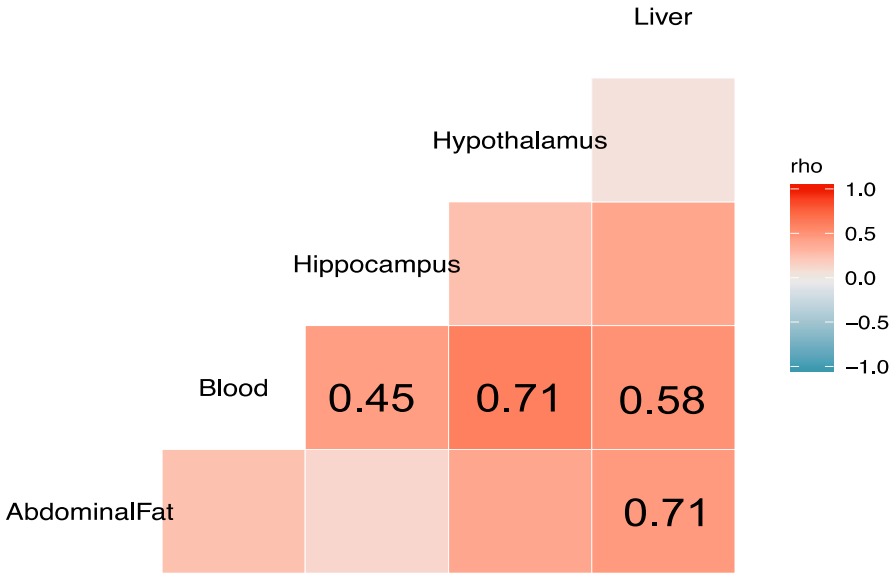

Figure 2. Pairwise correlations of mean tail moment values among individuals, regardless of cohort (n=21). All rho values indicated have p values < 0.05.

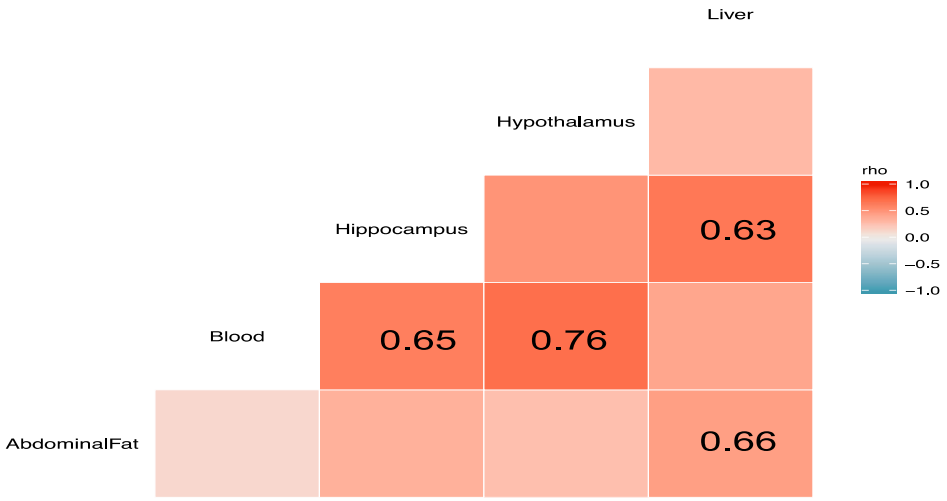

Figure 3. Pairwise correlations of mean tail moment values among captive individuals (n=13). All rho values indicated have p values < 0.05.

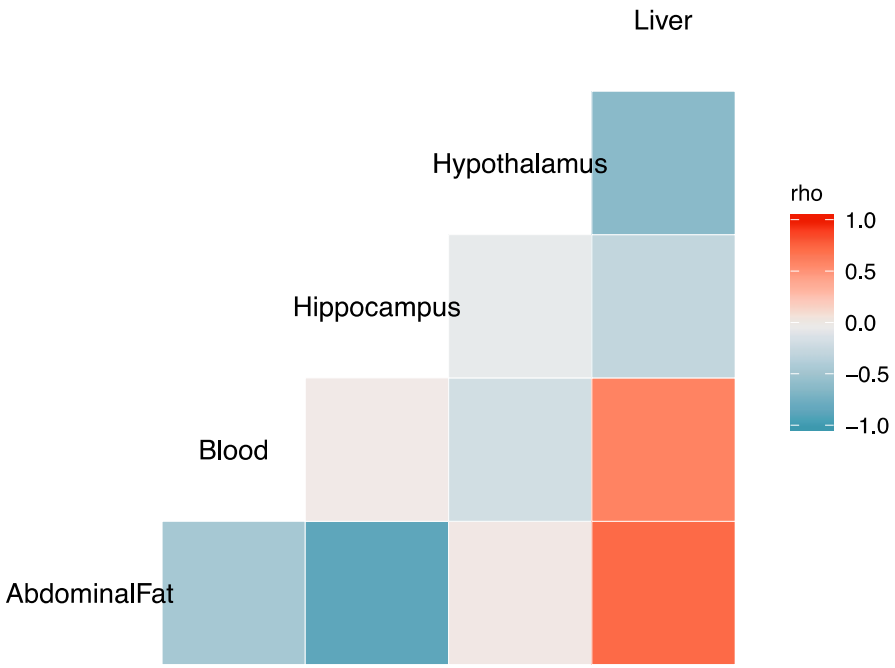

Figure 4. Pairwise correlations of mean tail moment values among wild individuals (n=9). No rho values have p values < 0.05.

4. Discussion

4.1 Comparisons across Tissue Type within Cohorts

The lack of significant differences in DNA damage across tissue types within cohorts (Fig. 1) is not surprising considering the lack of stress-protocols to which the tissues could differentially respond. Moreover, all mean tail moment values fit within the range of no and low damage standardized damaged cells (tail moment < ~ 40 pixels), suggesting low basal levels of DNA damage across tissues in unstressed birds regardless of captivity status. The data for the summer wild and summer captives suggest minimal differences between free-living and captive house sparrows. This may be promising for the use of this metric in captive populations with the hope of understanding wild populations considering other parameters such as plasma glucocorticoids have been observed to be profoundly affected by captivity. For example, captive white crowned sparrows baseline and stress-induced plasma glucocorticoids titers were twice as high as those in free-living sparrows (Romero and Wingfield, 1999) and Eastern red-spotted newts in captivity had baseline levels 20x as high as free-living newts (Berner et. al, 2013). However, consistently lower mean tail moments in the fall captives compared to the summer captives demonstrates the potential for inter-assay variation (Collins et. al, 2008; Langie et. al, 2015). As discussed in the statistical analysis section of the methods, this was attributed to the artificial registration of a higher DNA content in the comets by the OpenComet plug-in. Both third assay controls and samples had elevated DNA content, but DNA content in tail values for the samples were kept consistent while those of the controls were also elevated. It is likely that an upgrade of the computer and chip used with the fluorescent microscope to take and store images between the imaging of the first two assays and the third resulted in the artificial increase of fluorescence in the third assay comets. Because the standardized control cells are much larger and have more

defined tails they were less affected than smaller, low-damage sample cells. The increased fluorescence of heads and therefore increased DNA content in the "head" region is likely responsible for the variation observed. There is little biological premise for the variation between captive cohorts since all captives were caught around the same life history cycle and sacrificed while in captivity, under which other metrics like glucocorticoids had no seasonal affects (Berner et. al, 2013; Romero et. al, 2006).

4.2 Pairwise Correlations between Tissue Types and by Captivity Status

The positive, significant correlations observed in pairwise comparisons of tissue types when all birds were combined regardless of cohort suggest promising potential for the use of DNA damage as a correlate of DNA damage in the hippocampus ($r = 0.4584$, $S = 834$, $df = 20$, $p = 0.0380$, Fig. 2), hypothalamus ($r = 0.7157$, $S = 378$, $df = 19$, $p = 0.0005$, Fig. 2), and liver samples ($r = 0.5844$, $S = 640$, $df = 20$, $p = 0.0062$, Fig. 2). This supports the potential of use DNA damage in blood erythrocytes as a non-invasive proxy of damage elsewhere and suggests that the physiological effects of stress are generalized throughout the body. Moreover, we observed a positive, significant correlation between abdominal fat and liver samples ($r = 0.7147$, $S = 194$, $df = 15$, $p = 0.0025$, Fig. 2) which supports the rationale that both are being targeted as mobilizers of energy under the stress response (Romero and Wingfield, 2016). This correlation is particularly interesting as it suggests that the functions of these tissues and how they are targeted may be related to the ways they are interacting with and ultimately affected by chemical mediators of the stress response. This seems intuitive, but conflicts with the lack of uniformity in glucocorticoid receptor density and dynamics in metabolic tissues of house sparrows (Lattin and Romero, 2015). Similar observations were made in comparisons among only captives. This suggests that while captives from the third assay had reduced mean tail moments, the patterns

across tissues were not significantly changed. This further supports the rationale that the lower mean tail moments are the result of a systemic confounding variable. Interestingly, no significant correlations were detected when looking within solely the wild cohort. This may be due to the reduced sample size of birds (n=8) and further reduced samples size of abdominal fat tissue (n=5) due to lower fat stores in summer birds. However, it is surprising that correlations were so low and some were even negative, suggesting the potential for opposite relationships (though not statistically significant).

Ultimately, these data are but a portion of the greater picture that these experiments were meant to provide, but they illuminate the likelihood of correlations between blood and other tissues as well as among metabolic tissues that are similar targets of the stress pathways. However, they also demonstrate the potential for inter-assay variation and potential differences from what other metrics of stress such as glucocorticoids titers suggest. These very differences and the further study of them may be what set DNA damage apart as a new and rising measure of stress, but it will more likely solidify itself as another key piece of the clearly multi-faceted and complex stress phenotype.

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References

Baquedano, E., García-Cáceres, C., Diz-Chaves, Y., Lagunas, N., Calmarza-Font, I., Azcoitia, I., . . . Frago, L. M. (2011). Prenatal Stress Induces Long-Term Effects in Cell Turnover in the Hippocampus-Hypothalamus-Pituitary Axis in Adult Male Rats (Changes in Cell Death Induced by Prenatal Stress). PLoS ONE, 6(11), e27549. doi:10.1371/journal.pone.0027549

Berner, N. J., Heil, L. A., & Romero, L. M. (2013). Seasonal Variation in Corticosterone in Free-Living and Captive Eastern Red-spotted Newts Notophthalmus viridescens viridescens. Journal of Herpetology, 47(3), 466-470. doi:10.1670/12-069

Collins, A., Oscoz, A. A., Brunborg, G., Gaivao, I., Giovannelli, L., Kruszewski, M., . . . Stetina, R. (2008). The comet assay: topical issues. Mutagenesis, 23(3), 143-151. doi:10.1093/mutage/gem051

Dickens, M. J., & Romero, L. M. (2013). A consensus endocrine profile for chronically stressed wild animals does not exist. General and Comparative Endocrinology, 191, 177.

Dollé, M. E., Giese, H., Hopkins, C. L., Martus, H. J., Hausdorff, J. M., & Vijg, J. (1997). Rapid accumulation of genome rearrangements in liver but not in brain of old mice. Nature genetics, 17(4), 431. doi:10.1038/ng1297-431

Fischer, C. P., Wright-Lichter, J., & Romero, L. M. (2018). Chronic stress and the introduction to captivity: How wild house sparrows (Passer domesticus) adjust to laboratory conditions. General and Comparative Endocrinology, 259, 85-92. doi:10.1016/j.ygcen.2017.11.007

Flint, M. S., Baum, A., Chambers, W. H., & Jenkins, F. J. (2007). Induction of DNA damage, alteration of DNA repair and transcriptional activation by stress hormones. Psychoneuroendocrinology, 32(5), 470-479. doi:10.1016/j.psyneuen.2007.02.013

Gormally, B. M. G., Estrada, R., Yin, H., & Romero, L. M. (2019). Recovery from repeated stressors: Physiology and behavior are affected on different timescales in house sparrows. General and Comparative Endocrinology, 282. doi:10.1016/j.ygcen.2019.113225

Gormally, B. M. G., Fuller, R., McVey, M., & Romero, L. M. (2019). DNA damage as an indicator of chronic stress: Correlations with corticosterone and uric acid. Comparative Biochemistry and Physiology, Part A, 227, 116-122. doi:10.1016/j.cbpa.2018.10.007

Gyori, B. M., Venkatachalam, G., Thiagarajan, P. S., Hsu, D., & Clement, M.-V. (2014). OpenComet: an automated tool for comet assay image analysis. Redox biology, 2(1), 457. doi:10.1016/j.redox.2013.12.020

Hara, M. R., Kovacs, J. J., Whalen, E. J., Rajagopal, S., Strachan, R. T., Grant, W., . . . Lefkowitz, R. J. (2011). A stress response pathway regulates DNA damage through β2-adrenoreceptors and β-arrestin-1. Nature, 477(7364), 349. doi:10.1038/nature10368

Hara, M. R., Sachs, B. D., Caron, M. G., & Lefkowitz, R. J. (2013). Pharmacological blockade of a β2AR-β-arrestin-1 signaling cascade prevents the accumulation of DNA damage in a behavioral stress model. Cell Cycle, 12(2), 219-224. doi:10.4161/cc.23368

Langie, S. A. S., Azqueta, A., & Collins, A. (2015). The comet assay: part, present, and future. Front. Genet., 6. doi:10.3389/fgene.2015.00266

Lattin, C. R., Keniston, D. E., Reed, J. M., & Romero, L. M. (2015). Are receptor concentrations correlated across tissues within individuals? A case study examining glucocorticoid and mineralocorticoid receptor binding. Endocrinology, 156(4), 1354. doi:10.1210/en.2014-1811

Lattin, C. R., & Romero, L. M. (2013). Seasonal variation in corticosterone receptor binding in brain, hippocampus, and gonads in House Sparrows (Passer domesticus). The Auk, 130(4), 591-598. doi:10.1525/auk.2013.13043

Lattin, C. R., & Romero, L. M. (2014). Chronic stress alters concentrations of corticosterone receptors in a tissue-specific manner in wild house sparrows (Passer domesticus). The Journal of experimental biology, 217(14), 2601. doi:10.1242/jeb.103788

Lattin, C. R., & Romero, L. M. (2015). Seasonal variation in glucocorticoid and mineralocorticoid receptors in metabolic tissues of the house sparrow (Passer domesticus). General and Comparative Endocrinology, 214, 95-102. doi:10.1016/j.ygcen.2014.05.033

Love, A. C., Lovern, M. B., & Durant, S. E. (2017). Captivity influences immune responses, stress endocrinology, and organ size in house sparrows (Passer domesticus). General and Comparative Endocrinology, 252, 18-26. doi:10.1016/j.ygcen.2017.07.014

Malandrakis, E. E., Dadali, O., Golomazou, E., Kavouras, M., Dailianis, S., Chadio, S., . . . Panagiotaki, P. (2016). DNA damage and differential gene expression associated with physical stress in gilthead seabream (Sparus aurata). General and Comparative Endocrinology, 236, 98-104. doi:10.1016/j.ygcen.2016.07.009

McEwen, B. S. (1999). Stress and hippocampal plasticity. Annual review of neuroscience, 22, 105.

McEwen, B. S., & Wingfield, J. C. (2003). The concept of allostasis in biology and biomedicine. Hormones and Behavior, 43(1), 2-15. doi:10.1016/S0018-506X(02)00024-7

Olive, P., & Banáth, J. (2006). The comet assay: a method to measure DNA damage in individual cells. Nature Protocols, 1(1), 23-29. doi:10.1038/nprot.2006.5

Olive, P. L. (2002). The comet assay. An overview of techniques. Methods in molecular biology (Clifton, N.J.), 203, 179.

Pellettieri, J., & Sánchez Alvarado, A. (2007). Cell turnover and adult tissue homeostasis: from humans to planarians. Annual review of genetics, 41, 83.

Rigamonti, A., Brennand, K., Lau, F., & Cowan, C. A. (2011). Rapid Cellular Turnover in Adipose Tissue (Turnover in Adipose Tissue). PLoS ONE, 6(3), e17637. doi:10.1371/journal.pone.0017637

Romero, L. M., & Wingfield, J. C. (1999). Alterations in hypothalamic–pituitary–adrenal function associated with captivity in Gambel's white-crowned sparrows (Zonotrichia leucophrys gambelii). *Comparative Biochemistry and Physiology, Part B, 122*(1), 13-20. doi:10.1016/S0305-0491(98)10161-X

Romero, L. M. (2002). Seasonal changes in plasma glucocorticoid concentrations in free-living vertebrates. In (Vol. 128, pp. 1-24).

Romero, L. M., Cyr, N. E., & Romero, R. C. (2006). Corticosterone responses change seasonally in free-living house sparrows (Passer domesticus). *General and Comparative Endocrinology, 149*(1), 58-65. doi:10.1016/j.ygcen.2006.05.004

Romero, L. M., Dickens, M. J., & Cyr, N. E. (2009). The reactive scope model — A new model integrating homeostasis, allostasis, and stress. Hormones and Behavior, 55(3), 375-389. doi:10.1016/j.yhbeh.2008.12.009

Romero, L. M., Platts, S. H., Schoech, S. J., Wada, H., Crespi, E., Martin, L. B., & Buck, C. L. (2015). Understanding stress in the healthy animal - potential paths for progress. Stress, 18(5), 491-497. doi:10.3109/10253890.2015.1073255

Romero, L. M. (2016). *Tempests, poxes, predators, and people : stress in wild animals and how they cope*: Oxford ; New York : Oxford University Press.

Romero, L. M., & Gormally, B. M. G. (2019). How Truly Conserved Is the “Well-Conserved” Vertebrate Stress Response? Integrative and Comparative Biology, 59(2), 273-281. doi:10.1093/icb/icz011

Roth, T. C., Ladage, L. D., Freas, C. A., & Pravosudov, V. V. (2012). Variation in memory and the hippocampus across populations from different climates: a common garden approach. Proceedings of the Royal Society B, 279(1727), 402-410. doi:10.1098/rspb.2011.1020

Sapolsky, R. M., Romero, L. M., & Munck, A. U. (2000). How do glucocorticoids influence stress responses? Integrating permissive, suppressive, stimulatory, and preparative actions. Endocrine reviews, 21(1), 55. doi:10.1210/edrv.21.1.0389

Wingfield, J. C., Vleck, C. M., & Moore, M. C. (1992). Seasonal changes of the adrenocortical response to stress in birds of the Sonoran desert. Journal of Experimental Zoology, 264(4), 419-428. doi:10.1002/jez.1402640407