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Background DNA damage is higher in summer than winter in both free-living and captive birds

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Abstract

Although stress can cause overall damage to the genome, it is currently unknown whether normal background damage to DNA varies throughout the annual cycle. If DNA damage did vary seasonally, it would have major implications on environmental-genomic interactions. We measured background DNA double-stranded breaks using the neutral comet assay in five tissues (nucleated red blood cells, abdominal fat, hippocampus, hypothalamus, and liver) in four cohorts of house sparrows (*Passer domesticus*): free-living summer, captives on a summer light cycle, free-living winter, and captives on a winter light cycle. The experiment was designed to answer three questions: (1) Is red blood cell DNA damage representative of other tissues? (2) Is DNA damage in captive birds representative of DNA damage in free-living birds? (3) Does DNA damage show seasonality? We found that (1) blood is a representative tissue, (2) captive animals are representative of free-living animals, and (3) DNA damage is higher in the summer than in the winter. These data indicate that red blood cells can be an index of DNA damage throughout the body and that background levels of DNA damage show substantial seasonal variation. The latter result suggests the possibility that underlying molecular mechanisms of DNA damage and/or repair also change seasonally.

KEYWORDS
comet assay, DNA damage, house sparrow, seasonality, stress

1 | INTRODUCTION

Although glucocorticoid release is a classical stress index in animals because of its far-reaching effects, it provides a limited snapshot of the overall profile of an animal (Gormally, Estrada et al., 2019, Gormally, Fuller et al., 2019; Romero & Beattie, 2021; Romero et al., 2015; Sapolsky et al., 2000). DNA damage might serve as a better index, because it appears to be an integrative downstream measure of an animal's interaction with its environment via changes caused by both glucocorticoid and catecholamine exposure (Casagrande et al., 2020; Costantini et al., 2011; Flint et al., 2007; Hara et al., 2011). For example, DNA damage increases in response to acute (Flint et al., 2007; Gormally et al., 2020; Malandrakis et al., 2016) and chronic stress (Gormally, Fuller et al., 2019; Herborn et al., 2014; Sohn et al., 2012). Moreover, because avian species commonly used in studying the stress response, such as house sparrows (*Passer domesticus*) (Fischer et al., 2018; Lattin & Romero, 2013; Love et al., 2017), have nucleated erythrocytes, the relatively noninvasive acquisition of blood samples can allow for repeated measures of DNA damage (Gormally et al., 2020; Gormally, Fuller et al., 2019; Naz et al., 2020). However, the biological relevance of DNA damage in erythrocytes, which are not nucleated in other taxa, is not clear. It is currently unknown

whether DNA damage measured in erythrocytes is representative of DNA damage elsewhere in the body. In vitro studies have linked DNA damage to glucocorticoid exposure (Flaherty et al., 2017; Flint et al., 2007), suggesting that stress-induced DNA damage will be greatest in tissues that have the most glucocorticoid receptors. Because glucocorticoid receptors vary across tissue types in house sparrows (Lattin et al., 2012), we predicted that DNA damage would also vary across tissue types.

Furthermore, glucocorticoid receptor numbers in house sparrows vary seasonally in some tissues but not others (Lattin et al., 2013). For example, glucocorticoid receptor density changed seasonally in subcutaneous fat, kidney, liver, gastrocnemius, and pectoral muscle, but not in omental fat (Lattin et al., 2015). However, none of those tissues shared a seasonal peak in receptor density, suggesting that seasonal receptor changes occur in a tissue-specific manner (Lattin et al., 2015). Because corticosterone titers are equivalent in the summer and winter in free-living house sparrows (Romero et al., 2006), we predicted that DNA damage would be equivalent in both seasons but might vary by tissue type.

To measure DNA damage, we used the comet assay (also known as single-cell gel electrophoresis) in which sample cells are diluted and embedded in agarose on a microscope slide. The cells are lysed and then the entire sample/agarose/slide is placed in an electrophoresis chamber and electrophoresed at a low voltage. Just as a typical gel electrophoresis, smaller fragments of DNA (samples with more double strand breaks) will travel further in the gel than more intact DNA. Once the agarose is dried, the slide is stained and imaged. The resulting fluoresced images appear as comets, with the more fluorescent and compact head containing larger fragments and the tail containing streaks of smaller, more mobile fragments. Therefore, longer tails would be expected in cells with greater DNA damage and fragmentation. We used a neutral comet assay (Olive & Banáth, 2006) to measure primarily double-strand breaks in our experiment as opposed to an alkaline comet assay, which measures both double- and single-strand breaks. Avian erythrocytes contain many alkali labile sites, which turn to strand breaks when treated with the alkaline electrophoresis buffer, thus inflating the measured damage (Bonisoli-Alquati et al., 2010; Galván et al., 2014; Gormally, Fuller et al., 2019). The neutral assay has been used in house sparrows to measure DNA damage in erythrocytes during both acute (Gormally et al., 2020) and chronic (Gormally, Fuller et al., 2019) stress.

We quantified DNA damage in house sparrows across five tissues involved in the stress response: abdominal fat (site 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 the hypothalamic-pituitary-adrenal axis), and the liver (primary metabolic target of glucocorticoids and catecholamines) (McEwen, 1999; reviewed in Romero & Wingfield, 2016). We compared damage in both wild and captive house sparrows during the summer and winter. To our knowledge, this is the first characterization of DNA damage levels in free-living house sparrows across seasons and tissue types.

2 | METHODS

2.1 | Sample collection

We caught adult house sparrows from the same site in Eastern Massachusetts in July 2019 ($n = 9$; 6M:3F) and January 2020 ($n = 9$; 5M:4F) with mist nets. Two further sets of house sparrows were caught in Eastern Massachusetts and allowed to acclimate to captivity for at least 4 weeks (Fischer et al., 2018). These birds were housed individually or in male–female pairs in cages (45 × 37 × 33 cm) on a 12L:12D light cycle to simulate summer ($n = 5$, 2M:3F) or 9L:15D light cycle to simulate winter ($n = 8$, 5M:3F). Water, seed, and grit were provided ad libitum. All procedures were approved by the Tufts Institutional Animal Care and Use Committee and were conducted in compliance with the Guidelines for Use of Wild Birds in Research (Fair et al., 2010)

For free-living birds, 20 µl of blood were collected from the alar vein within 2 min of capture and stored on ice. Birds were then anesthetized with isoflurane, 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 phosphate buffered saline (PBS; Ca^{2+} Mg^{2+} free). Before transferring to PBS, liver samples were cut and left to sit in a weigh boat filled with chilled PBS for ~1 min to remove excess blood. Sample collection was completed within 13 min of capture. Cell suspensions of blood were prepared by diluting 2 µl of whole blood into 800 µl of chilled PBS followed by two fivefold dilutions (100 µl of suspension into 400 µl of fresh PBS) (Gormally, Fuller et al., 2019). Abdominal fat, hippocampus, hypothalamus, and liver were minced in the original 1 ml of PBS using fine shears. The liver suspensions underwent one fivefold dilution (100 µl of suspension into 400 µl of fresh PBS). Captive birds were sampled in the same way, only they were captured from cages. We ran 148 samples in total from 31 birds. Seven birds did not have enough abdominal fat to harvest and assay (5 from the summer/free-living group, 1 from the winter/free-living group, and 1 from the winter/captive group).

2.2 | Comet assay

A 30 µl aliquot of each suspension was diluted in 300 µl of warm (37°C), low-melting agarose (R&D Systems Cat. No. 425005001). After vortexing, 30 µl of sample were plated via pipette such that the gel spread evenly throughout the well of the CometSlide (R&D Systems Cat. No. 425250001). Within seasons, samples were sorted randomly such that an individual's five tissues were spread across multiple slides to minimize order effects. Samples were plated in duplicate and complete slides were stored for 30 min at 4°C to solidify the gel. Slides were then treated with cold lysis buffer (R&D Systems Cat. No. 425005001) mixed with 10% DMSO for 1 h at 4°C, followed by equilibration in electrophoresis buffer (300 mM sodium acetate, 10 mM Tris base, pH 10) for 30 min at 4°C (Gormally, Fuller et al., 2019; Olive & Banáth, 2006).

Slides were then electrophoresed in fresh electrophoresis buffer for 30 min (21V, 4°C), washed twice in chilled dH_20 for 5 min to neutralize, and soaked in 70% ethanol for 5 min. Lastly, they were heated at 37°C for 20–40 min to dry slides of excess liquid and evaporate gels. Dried slides were stored with desiccant in a cool, dark place until staining and imaging. The entirety of the assay was performed under low-light conditions to reduce DNA-damage by UV light (Gormally, Fuller et al., 2019; Olive & Banáth, 2006). Standardized damaged cells (R&D Systems Cat. No. 4257010NC) were plated and run with samples during each assay. These standardized cells allowed for an estimation of variation between assays and to normalize data if necessary (Gormally, Fuller et al., 2019). The data collected required three assays, but five runs of electrophoresis (due to a limit of five slides in the electrophoresis chamber). The inter-assay variability was 31% and the intra-assay variability was 22%. To normalize the data using the control cells, we averaged each control cell well across plates in each assay. We then computed the fold change from the assay with the lowest control cells (the summer assay) and finally divided all values by the computed fold change (e.g., the control cells were 1.7 times higher in the winter captive assay than they were in the summer assay, so all winter captive values were divided by 1.7).

2.3 | Slide staining, microscopy, and image processing

The slides were stained with SYBR™ Gold (Invitrogen Cat. No. S11494) for 30 min. Excess stain was removed by washing slides with chilled dH_2O. Slides were then dried at 37°C for approximately 20 min and imaged using a fluorescent microscope with a green fluorescent protein filter at x10 objective. The Fiji plug-in OpenComet was used to detect comets in the images taken and erroneous detections were manually removed by an investigator blinded to sample source (Gyori et al., 2014). We used the "TailMoment" metric produced by OpenComet, which is % tail DNA × tail length. There is no consensus on the best parameter to use when analyzing comet images, but the most recent study on DNA damage in house sparrow erythrocytes (Gormally et al., 2020) used the tail moment parameter.

2.4 | Statistics

All statistical analyses were conducted in RStudio (Team, 2021). The following models were tested for homogeneity of variance using Levene's test (car package, Fox & Weisberg, 2011) and square-root-transformed according to Tukey's Ladder of Powers. We then ran a linear model with season, captivity state, tissue, sex, and each interaction, in which sex was not significant and subsequently removed. The final model was comprised of season, captivity state, tissue, and each interaction. Because harvesting tissues required terminal procedures, the birds in each season/captivity state group were unique, which means that we could not use a repeated-measures statistical analysis.

Cohen's *d* effect size was calculated using the "cohen.d" function of the "effsize" package (Torchiano, 2020).

3 | RESULTS

The overall model indicated that season ($F_1 = 60.35$, $p < 0.0001$) significantly affected DNA damage. In contrast, captivity state ($F_1 = 1.11$, $p = 0.29$), tissue ($F_4 = 1.86$, $p = 0.12$), the season x captivity state interaction ($F_1 = 2.09$, $p = 0.15$), the seasonxtissue ($F_4 = 1.43$, $p = 0.23$), the captivity state x tissue interaction ($F_4 = 2.09$, $p = 0.08$), and the season x captivity state x tissue interaction ($F_4 = 12.01$, $p = 0.10$) did not. DNA damage in the summer was higher than in the winter, with a difference in tail moment of 24.44 (Cohen's $d = 2.61$). The adjusted R^2 of the model is 0.70.

4 | DISCUSSION

4.1 | Is DNA damage in blood representative of DNA damage in other tissues?

No tissue (abdominal fat, hippocampus, hypothalamus, or liver) significantly differed from blood (Figure 1, comparing across panels), indicating that DNA damage in blood is representative of the other four tissues in house sparrows. This finding is important because blood sampling allows minimally invasive repeated-measures study designs from an individual animal, whereas measuring damage in other tissues requires euthanasia. Interestingly, blood was not a representative tissue compared to liver and gills for free-living chub (*Squalius cephalus L.*) (Sunjog et al., 2014). However, the study on chub used an alkaline comet assay (which measures single strand breaks [Lu et al., 2017]) and different analysis outputs (tail intensity and olive tail moment, instead of tail moment). We suggest that researchers validate blood as a representative tissue in their study species. Further research is also needed to determine if blood is a representative tissue in other contexts, such as chronic stress.

4.2 | Is DNA damage in captive birds representative of DNA damage in free-living birds?

The effect of captivity on physiology is highly species and system specific (Calisi & Bentley, 2009; Fischer & Romero, 2019; Fischer et al., 2018). Introducing house sparrows to captivity results in weight decreases and initial increases in corticosterone levels that level-off to a "new normal" (Fischer et al., 2018; Gormally, Fuller et al., 2019). One study tracked DNA damage in house sparrows repeatedly through the introduction to captivity (in April) and found an increase by 3 days of captivity that remained elevated through 27 days (Gormally, Fuller et al., 2019). Birds used in our study had been in

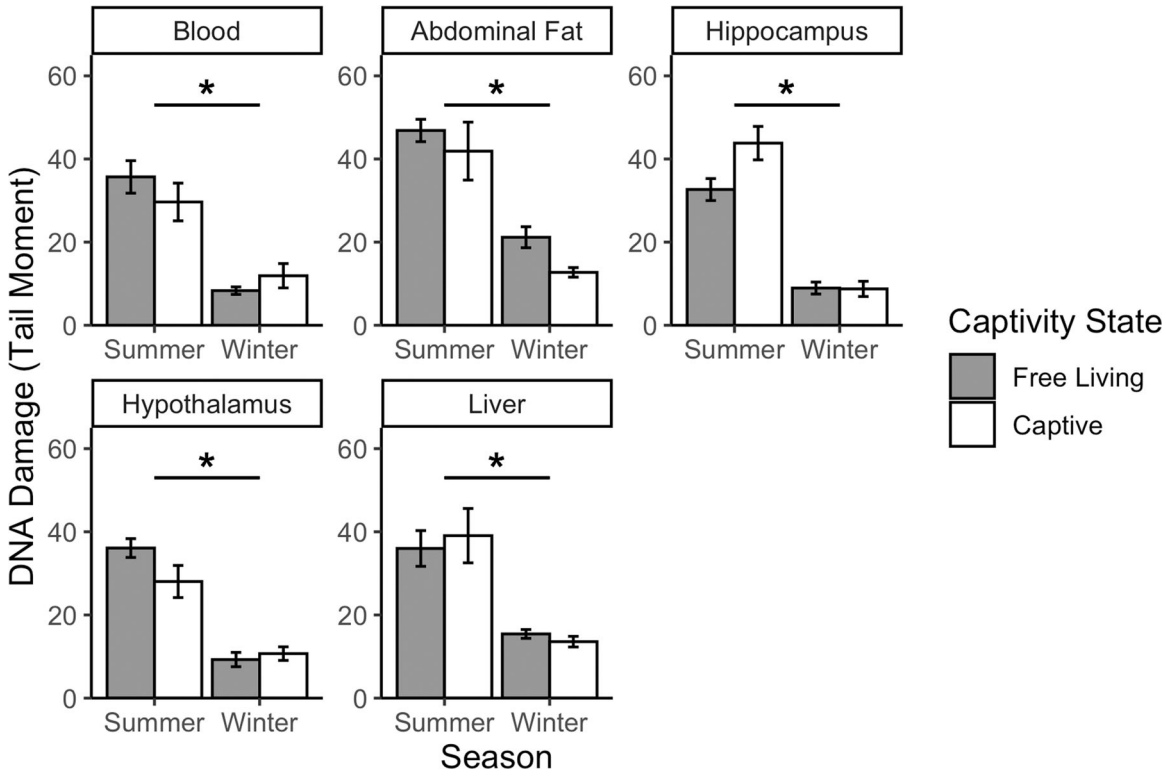

FIGURE 1 DNA damage in free-living and captive house sparrows in winter and summer. Across tissues, DNA damage in the winter is lower than in the summer. There were no significant differences among the tissues or captivity states within a season. *$p < 0.05$.

captivity for at least 4 weeks but showed no difference in DNA damage compared to their wild counterparts in any of the tissues measured (Figure 1, comparing colors within each column grouping). While we would've expected similar results to Gormally, Fuller et al., 2019; our study precluded measuring DNA damage in the same bird pre- and post-captivity which may have contributed to the difference. Despite this difference, the overall conclusion is that DNA damage in captive birds is representative of DNA damage in free living birds.

4.3 | Does DNA damage change seasonally?

In every tissue measured, DNA damage in the winter was lower than damage in the summer (Figure 1, comparing column groupings within each panel). This contradicts our initial hypothesis that DNA damage would be equivalent in the summer and winter due to previously observed equivalent corticosterone levels across seasons (Romero et al., 2006). Earlier studies suggested that DNA damage and corticosterone titers would be coupled because acute increases in corticosterone increased DNA damage in vitro (Flint et al., 2007) and a chronic rise in corticosterone paralleled with a chronic rise in DNA damage in vivo (Gormally, Fuller et al., 2019). However, Gormally, Fuller et al. (2019) noted that absolute levels of corticosterone and DNA damage were not correlated, indicating that two different mechanisms must be at play. Our data also supports distinct mechanisms because DNA damage, but not corticosterone, showed seasonal differences.

To our knowledge, this is the only study to measure specifically double stranded DNA breaks across seasons. Other studies (Betti et al., 1995; Dušinská et al., 2002; Gerić et al., 2018; Møller et al., 1998; Sunjog et al., 2014; Verschaeve et al., 2007) that have compared DNA damage across seasons have used an alkaline comet assay, which detects more types of DNA damage (Lu et al., 2017). In chub erythrocytes (Sunjog et al., 2014), DNA damage is higher in the winter than in the summer—the opposite pattern to this study. Studies in seasonality of human lymphocyte and monocyte DNA damage (Betti et al., 1995; Dušinská et al., 2002; Gerić et al., 2018; Møller et al., 1998; Verschaeve et al., 2007) do not show consistent results. Additionally, it is difficult to compare seasonality in humans and free-living animals because humans have altered many aspects of their environment. Given those caveats, it is still interesting that previously documented seasonal patterns of DNA damage are different than our study.

Not only is there elevated DNA damage in the summer in free-living birds, this elevation persists in captivity. There are many seasonal habitat differences that might cause physiological changes in the wild, such as reproduction or changes in temperature, daylight, and the availability of food and shelter. This is in contrast, however, to the captive data in our study. Our birds do not reproduce in captivity (and are not given a gradual lengthening of daylength to trigger reproduction). The only difference between captive summer

and winter birds was the light cycle and yet there was still higher DNA damage in the "summer." Studies in humans (Dušinská et al., 2002; Gerić et al., 2018; Møller et al., 1998; Verschaeve et al., 2007) have suggested an increase in solar radiation might be the culprit for increased DNA damage in the summer, however it is unlikely that sun rays could penetrate a birds skin and feathers. Thus, it is unclear why a change in daylight might alter DNA damage, but this is an exciting avenue for future research.

5 | CONCLUSIONS

Free-living house sparrows show surprising, and hitherto undocumented, seasonal differences in baseline DNA damage that is, consistent across tissues and persists in captivity. The reason for this variability is not presently known, but it suggests the intriguing possibility that underlying molecular mechanisms of either DNA damage or DNA repair also change seasonally.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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