

ORIGINAL CONTRIBUTION

The Effect of a Combined Fast and Chronic Stress on Body Mass, Blood Metabolites, Corticosterone, and Behavior in House Sparrows (*Passer domesticus*)

Ursula K. Beattie*, Michelle C. Ysrael, Sarah E. Lok, and L. Michael Romero

Department of Biology, Tufts University, Medford, MA, USA

One aspect of the Reactive Scope Model is wear-and-tear, which describes a decrease in an animal's ability to cope with a stressor, typically because of a period of chronic or repeated stressors. We investigated whether wear-and-tear due to chronic stress would accelerate a transition from phase II to phase III of fasting. We exposed house sparrows (*Passer domesticus*) to three weeks of daily fasts combined with daily intermittent repeated acute stressors to create chronic stress, followed by two weeks of daily fasts without stressors. We measured circulating glucose, β -hydroxybutyrate (a ketone), and uric acid in both fasted and fed states. We expected birds to be in phase II (high fat breakdown) in a fasted state, but if wear-and-tear accumulated sufficiently, we hypothesized a shift to phase III (high protein breakdown). Throughout the experiment, the birds exhibited elevated β -hydroxybutyrate when fasting but no changes in circulating uric acid, indicating that a transition to phase III did not occur. In both a fasted and fed state, the birds increased glucose mobilization throughout the experiment, suggesting wear-and-tear occurred, but was not sufficient to induce a shift to phase III. Additionally, the birds exhibited a significant decrease in weight, no change in corticosterone, and a transient decrease in neophobia with chronic stress. In conclusion, the birds appear to have experienced wear-and-tear, but our protocol did not accelerate the transition from phase II to phase III of fasting.

INTRODUCTION

The Reactive Scope Model [1] is a model of stress that focuses on the physiological mediators involved in the stress response. The model describes four potential ranges of physiological mediators: predictive homeostasis (ranges of physiological mediators involved in

responding to daily or seasonal changes); reactive homeostasis (ranges of physiological mediators in response to an unpredictable change); homeostatic failure (ranges of the physiological mediators too low to sustain homeostatic mechanisms); and homeostatic overload (ranges at which the physiological mediators themselves becomes pathological). Wear-and-tear (similar to an increase in

*To whom all correspondence should be addressed: Ursula Beattie, Department of Biology, Tufts University, 200 College Avenue, Medford, MA 02155; Email: Ursula.Beattie@tufts.edu; ORCID iD: 0000-0002-7131-3712.

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allostatic load [2]) occurs when physiological mediators remain high or low for too long and is modeled by a decrease in the threshold between reactive homeostasis and homeostatic overload. Wear-and-tear thus decreases an organism's ability to cope with a stressor. The purpose of this study was to investigate how chronic stress induces wear-and-tear on metabolic energy usage in captive house sparrows (*Passer domesticus*).

Perhaps the most important environmental variable affecting energy usage is energy availability. The lack of energy input can be broken down into both short-term (eg, an overnight fast) and long-term (eg, starvation) durations of low food availability. Vertebrates use different mechanisms to cope with these different periods, but only involuntary fasting is thought to require a stress response [3,4] and thus modeled by reactive scope. The process of involuntary prolonged fasting can be separated into three phases: phase I involves primarily carbohydrate metabolism (and uses coping mechanisms similar, if not identical to a short-term fast), phase II is primarily fat metabolism, and phase III is primarily protein metabolism [3,5]. The transition between phases is a consequence of exhausting a nutrient pool. If refeeding does not occur by the end of phase III, the animal will die. The amount of time that an animal can fast is highly species dependent, with house sparrows typically unable to fast longer than 24-32 hours [6,7]. In the context of reactive scope, phase I and phase II can be considered part of both predictive homeostasis and reactive homeostasis, depending on the species and food availability. Phase III involves breaking down important proteins, including muscle mass, and thus reflects the homeostatic overload range [3].

Although the physiological responses to both food restriction and chronic stress have been studied extensively, it is not currently known how homeothermic vertebrates cope with a combined challenge. We exposed house sparrows to a nightly 18-hour fast, which we predicted would push house sparrows into phase II of fasting. At the conclusion of each daily fast, we therefore predicted that the birds would have low-to-mid levels of carbohydrate breakdown, high levels of fat breakdown, and low levels of protein breakdown, indicating that birds were in phase II of fasting. After refeeding, we predicted that birds would replenish their carbohydrates, have low levels of fat breakdown, and high levels of protein breakdown due to dietary protein [8]. On top of this daily fast, we layered chronic stress by subjecting the birds to an established chronic stress protocol that exposed birds to four rotating mild psychological stressors 3 times per day for three weeks [9,10]. If the chronic stress period caused the birds to experience a decreased ability to cope with stress ("wear-and-tear"), we predicted the birds would enter into phase III of fasting earlier than they normally would. If this were to happen, we would expect low-to-

mid levels of carbohydrate and fat breakdown and high levels of protein breakdown at the end of the daily fast. Additionally, we would expect a spike in corticosterone, which facilitates the transition into phase III [5,11-13].

We monitored metabolism of carbohydrates, fats, and proteins by measuring plasma concentrations of glucose, β -hydroxybutyrate, and uric acid, respectively. β -hydroxybutyrate is a type of ketone body, which is converted from fat stores in the liver and can be used as a source of energy in phase II of fasting [14,15]. β -hydroxybutyrate has been suggested for use as a robust indicator of fasting in wild species [16]. Plasma uric acid is a waste product of protein breakdown which circulates in the bloodstream until it reaches the kidneys and is filtered out and expelled [14,17]. To measure these metabolites, we used human point-of-care devices, which are promising for use in field studies, where access to equipment, freezers, and labs are not available [18-20]. Additionally, these point-of-care devices require small amounts of blood (2-3 μ L), allowing for frequent sampling.

In addition to metabolites, we included two behavioral measures. Behavior is also sensitive to both food restriction [21,22] and chronic stress [23]. For example, fasted birds approach food more quickly despite the potential increased risk of predation [24]. We measured overall activity (perch hops) and neophobia (fear of the new) by quantifying latency to approach a food dish with and without the presence of a novel object. Increased total activity could mean birds have become more anxious and are displaying food seeking behavior [21]. If chronically stressed birds became more fearful, latency to approach a novel object would increase; conversely, if an animal became more desperate for food, and thus bolder, latency to approach would decrease.

House sparrows are an established model species for studies on wild animal stress physiology [25] and are known to undergo the classic three phases of fasting [6]. Furthermore, we chose a captive wild species because captive-bred animals are functionally different animals than their wild counterparts [26]. To our knowledge, this is the first study that combines a psychological chronic stress and daily food restriction in a wild homeothermic vertebrate in order to understand how the two stressors combine to modulate metabolism.

METHODS

House sparrows were caught using mist nets in February and March of 2019 in eastern Massachusetts. The birds were doubly housed (M/F or F/F) in cages (45 cm x 37 cm x 33 cm) in a single room and allowed to acclimate to captivity for approximately 7 months before the experiment began. Two weeks prior to the start of the ex-

periment, birds were transferred to individual cages and the light cycle was changed to reflect the short amount of daylight in winter in Massachusetts (9L:15D, which corresponded to the lights being on from 9:00am to 6:00pm). The birds had *ad libitum* food (a mix of sunflower seeds and millet) and water until the start of the experiment, upon which food was restricted, but water remained *ad libitum*. The study group consisted of 7 females and 9 males, but 4 females and 4 males were randomly selected to be euthanized after the third week of the experiment for another project. Despite reducing the sample size to 8 in the second portion of the experiment, we still have sufficient power for the study. A power analysis indicates a 95% chance of detecting a 50% difference in means with 20% variability with 8 birds in an experimental group using an alpha of 0.05. All experiments were performed according to the guidelines for use of wild birds in research [27] and approved by the Tufts University Institutional Animal Care and Use Committee.

Point-of-Care Device Validation

Before the main experiment began, point-of-care devices were validated directly against standard bench assays for each metabolite. For the validation studies, none of the birds used were also used in the main experiment. In short, birds were bled by puncturing the alar vein with a 26-gauge needle and approximately 60 μ L of blood was collected in a heparinized capillary tube. Immediately after bleeding, each sample was used to quantify the metabolites in a standard bench assay and a point-of-care device. $N=22$ birds for glucose comparison, $n=16$ for ketone comparison, and $n=15$ for uric acid comparison. The point-of-care devices used were Precision Xtra Blood Glucose and Ketone Monitoring System (Cat. No. 98814-65) with the glucose (Cat. No. 99838-65) and ketone (Cat. No. 70745-BX) test strips and the UASure Blood Uric Acid Meter (Cat. No. U3003). The standard bench assays used for comparison were the Sigma-Aldrich Glucose (HK) Assay Kit (Cat. No. GAHK20), Sigma-Aldrich Ketone Body Assay (Cat. No. MAK134), and the Molecular Probes Amplex Red Uric Acid/Uricase Assay Kit (Cat. No. A22181). The ketone and uric acid assays were performed following the manufacturers' protocols. To scale the glucose assay for use in a 96-well plate, we used 100 μ L of assay reagent and 20 μ L of diluted blood. The assay requires samples to have between 0.05 and 50 μ g of glucose for accurate measurements, so blood was diluted 1:13 in deionized water prior to being added to the assay reagent. All other aspects of the glucose assay were performed according to the manufacturer's protocol.

As an additional validation experiment, we tested whether each point-of-care device would detect an expected difference. Fed birds should have higher glucose

and uric acid (because of the high levels of uric acid in sunflower seeds [8]) compared to fasted birds, whereas fasted birds should have higher levels of ketones as they metabolize fat. We removed the birds' food in the evening, took a blood sample the next morning before food addition, and again one hour after food addition. Glucose, ketones, and uric acid were quantified using the point-of-care devices described above ($n=39$ birds for all comparisons).

Experimental Design

The five-week experiment consisted of three weeks of a previously validated chronic stress protocol [9,10] and two weeks post-chronic stress (Figure 1). To induce chronic stress, the birds were subjected to three 30-minute stressors per day. The first stressor was at randomly chosen times between 9:00am and 10:00am, the second stressor between 1:00pm and 4:30pm, and the third stressor between 5:30pm and 6:30 pm. These time intervals were chosen so stressor exposure would be unpredictable yet not interfere with sample collection and food replacement. The stressors included playing the radio in the bird room, reading to the birds, running a pen along the cages, and rolling the cage racks around the room. The type of stressor was also randomized. All aspects of the post-chronic stress period were the same as the chronic stress protocol, except for the absence of the stressors.

Each day, the birds were given food *ad libitum* at 11:00am and it was taken away at 5:00pm. When food was taken away each day, the cage liners were also changed, to eliminate birds eating food that had fallen out of the food dish. Every 2 to 3 days, two blood samples were taken from each bird: one before food addition (10:30am) and one after 1 hour of food being added (12:00 noon), to represent a fasted and fed state, respectively. Due to ethical restrictions on the amount of blood that can be drawn from a house sparrow, we could not take a blood sample for corticosterone quantification at every timepoint. Metabolites were measured for every blood sample, but half of the birds ($n=8$) had blood drawn for corticosterone quantification in a fasted state, and the other half of the birds ($n=8$) had corticosterone quantified in a fed state. Approximately 15 μ L of blood was drawn when only metabolites were to be measured and approximately 60 μ L of blood was drawn when both corticosterone and metabolites were to be measured. A total of 75 μ L of blood was taken from every bird on any given bleed day. After each fed blood sample, the birds were placed in opaque cloth bags until all birds were bled (within 3-5 min). At that point, the birds were weighed and the blood samples placed on ice until processing (see *Metabolite Analysis* and *Corticosterone Analysis* sections). At each blood sample timepoint during the three weeks of chronic stress

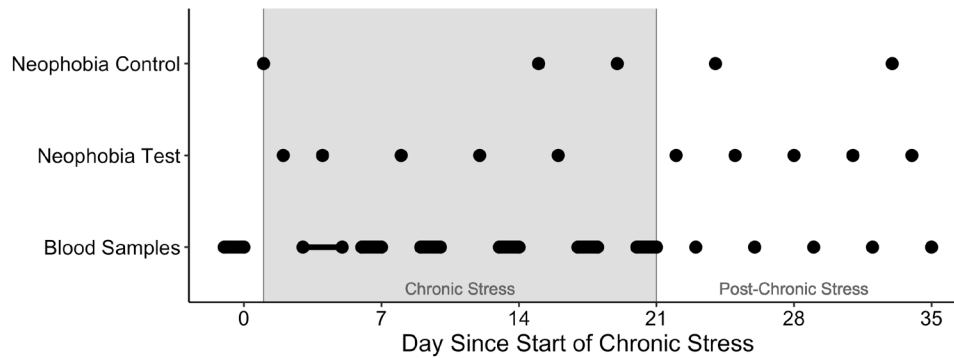


Figure 1. Timeline of datapoints taken. For blood samples taken during the chronic stress period, half of the birds were measured on consecutive days and averaged together (depicted by elongated dots), to ensure blood samples were taken in a timely manner after initial disturbance. Blood samples taken on days 3 and 5 were averaged together because no blood sample was taken on day 4 due to experimenter error (depicted by a narrow line connecting the dots on days 3 and 5). For days 0-21, $n=16$, for days 22-35, $n=8$.

(Figure 1), birds were bled over 2 days (8 birds on one day and 8 the next day) to ensure blood samples were taken within 5 minutes of entering the bird room [28]. The two sequential bleed days were then averaged together for analysis. Due to experimenter error, blood samples taken on days 3 and 5 were averaged together because no blood sample was taken on day 4. Within each blood sample day, birds were bled randomly (ie, birds were not bled in the same order each time they were bled).

An initial blood sample was taken from all birds in the 2 days before the start of the experiment. This sample was taken 3 hours after lights on with food available *ad libitum*. Fasting was initiated on the evening of the second initial sample day and the chronic stress protocol initiated the next morning.

Note that using wild-caught birds meant that a stress-free control treatment was not possible because the stressors inherent to captivity are unavoidable. Instead, we employed a repeated-measures design and previous studies indicate that birds do not acclimate to this paradigm of stress [eg, 9,29,30].

Metabolite Analysis

Glucose, β -hydroxybutyrate, and uric acid were measured on whole blood immediately after sample collection, using human point-of-care devices as described above. Approximately 1 μ L was needed for the glucose and β -hydroxybutyrate tests, and approximately 2 μ L was needed for uric acid. In instances where the value was higher than the detection limit of the device, a ceiling value of 500 mg/dL and 20 mg/dL was assigned for glucose and uric acid, respectively. Twelve out of 297 samples exceeded the glucose test, 33/297 samples exceeded the uric acid test, and no blood sample exceeded the ketone test.

Corticosterone Analysis

Immediately following metabolite analysis, the remainder of the whole blood was centrifuged at approximately 1200 g for 15 minutes. The extracted plasma was stored at -20°C until assayed with an established radioimmunoassay for corticosterone quantification [31]. In short, plasma samples were brought up to a volume of 200 μ L with distilled water and then spiked with 20 μ L of tritiated corticosterone (Perkin Elmer Cat. No. NET399250UC) to measure extraction efficiency. Steroids were extracted using dichloromethane, dried using nitrogen, and then resuspended with phosphate buffered saline with gelatin. Lastly, a standard curve and unknowns were assayed using the B3-163 antibody (Esoterix, Calabasas Hills, CA) and scintillation fluid (Ultima Gold Cat. No. 6013119) with adjustment made for extraction efficiency. Nineteen out of 139 samples fell below the detection limit of the assay, so were assigned the floor value calculated based on the volume of plasma added. Three assays were run in total, with the inter-assay and intra-assay variabilities of 12.0% and 2.7%, respectively.

Behavioral Tests

On days that blood samples were not taken (Figure 1), the birds were presented with novel objects (cocktail umbrella, straw, ribbon, plastic Easter egg, painted food dish, pipe cleaner, tulle, lei flower, Altoid box lid, Christmas ornament, or Mardi Gras beads) on or near their food dish, when food was presented at 11:00am. No bird got the same novel object more than once and birds were prevented from seeing future objects. Objects were randomized on each day and not all objects were used in one day (any given object trial could have been four different objects for four different cages). Videos were recorded on home security cameras from immediately before the experimenter entering the room and for 20 minutes after

food addition. After 20 minutes, the novel objects were removed from the cages, removed from the bird room, and cameras were turned off. Birds were presented with their food without a novel object on non-test days, but as a control, videos were also recorded for 20 minutes without objects (but still on a non-blood sample day). One day of control videos (day 11) was lost. An experimenter blinded to treatment analyzed each 20-minute video and recorded both the time it took for each bird to approach its food dish and total number of hops (defined as any movement throughout the cage). In instances where the bird did not approach its food dish within 20 minutes, the datapoints were assigned a ceiling value of 20 minutes. There were 38/184 instances in which a bird did not approach its food within 20 minutes.

Statistics

All statistical analyses were run in RStudio [32]. For all variables (weight, glucose, ketones, uric acid, corticosterone, perch hops, and neophobia), statistical analyses were run on the raw data, despite some metrics being graphed as percent change from the pre-chronic stress timepoint.

A linear regression was used to validate the point-of-care devices against the standard bench assays. The R^2 value was used to evaluate the correlation between the two methods. To compare values from the point-of-care devices in fed and fasted birds, we used a one-tailed paired t-test, because the same birds were sampled in a fasted state and in a fed state and we had expectations for what state should have higher metabolite levels in each case.

The effect of day on weight was analyzed using a linear mixed effect model ('lmer' function, lme4 package; ['lmer' function, lme4 package]) [33] with day as a fixed effect and bird identity as a random effect. Then, a Type III ANOVA (car package) [34] was run to test if the model was significant. Finally, the 'glht' function of the multcomp package [35] was used to compare each day of the experiment to the pre-chronic stress timepoint. In order to generate the comparisons, "day" had to be changed from a numerical class to a factor class. Finally, the same steps were employed on a subset of the data to compare the last day of the chronic stress period to each day of the post-chronic stress period.

For the other five variables, the linear mixed model required two fixed factors. For glucose, β -hydroxybutyrate, uric acid, and corticosterone, day and state (fasted/fed) were fixed effects and bird identity was a random effect in the model. If the Type III ANOVA was significant, the model was split into two models: one for fasted and one for fed (ie, one model with day as a fixed effect and bird identity as a random effect on all of the fasted data and another model with the same effects but on the fed data).

If the Type III ANOVAs on each of those models were significant, the 'glht' function was used to compare each day of the experiment to the pre-chronic stress timepoint within the fasted and fed states. Additionally, the data from the significant models were split to include only the last day of chronic stress and the post-chronic stress period. Another linear mixed model was run with day as a fixed effect and bird identity as a random effect. If the Type III ANOVA was significant, 'glht' was used to compare each day of the post-chronic stress period to the last day of chronic stress within fed and fasted.

The models for perch hops and neophobia were the same as glucose, β -hydroxybutyrate, uric acid, and corticosterone, except instead of fed vs fasted, the fixed effect was object vs no object.

For all variables, the effect of sex was checked on the initial linear mixed models, but because there was no effect of sex, or any interaction with sex, the factor was taken out for the remainder of the analyses. The lack of a sex effect on stress physiology is typical of studies on house sparrows [eg, 36-41].

For each step of the statistical analyses, the assumptions of a linear mixed effect model were checked using Levene's Test of homogeneity of variances and by visually inspecting the residual plots for each model. Data that did not pass the test were transformed according to Tukey's Ladder of Powers. The following transformations were used to ensure that each dataset passed Levene's test: square root of the full β -hydroxybutyrate dataset and the control neophobia dataset; the log of the fasted β -hydroxybutyrate dataset, the full uric acid dataset, and the full neophobia dataset; the reciprocal square root of the full neophobia dataset. All other datasets did not require transformations.

RESULTS

Point-of-Care Device Validation

The R^2 values for the linear regressions between the point-of-care devices and the bench assays for glucose, β -hydroxybutyrate, and uric acid are 0.54, 0.33, and 0.44, respectively (Figure 2A-C). Upon examining the graph for β -hydroxybutyrate, the two values in the bench assay below 0.7 mM were not consistent with the trend. After removing those two datapoints, the R^2 value becomes 0.67 and the trendline appears to be much more representative of the data (Figure 2B).

Glucose was significantly higher in fed birds compared to fasted birds ($t_{37} = 4.51, p < 0.0001$; Figure 2D). β -hydroxybutyrate was significantly higher in fasted birds compared to fed birds ($t_{37} = -6.94, p < 0.0001$; Figure 2E). Uric acid was significantly higher in fed birds compared to fasted birds ($t_{37} = 4.80, p < 0.0001$; Figure 2F).

Although the point-of-care devices appear to overes-

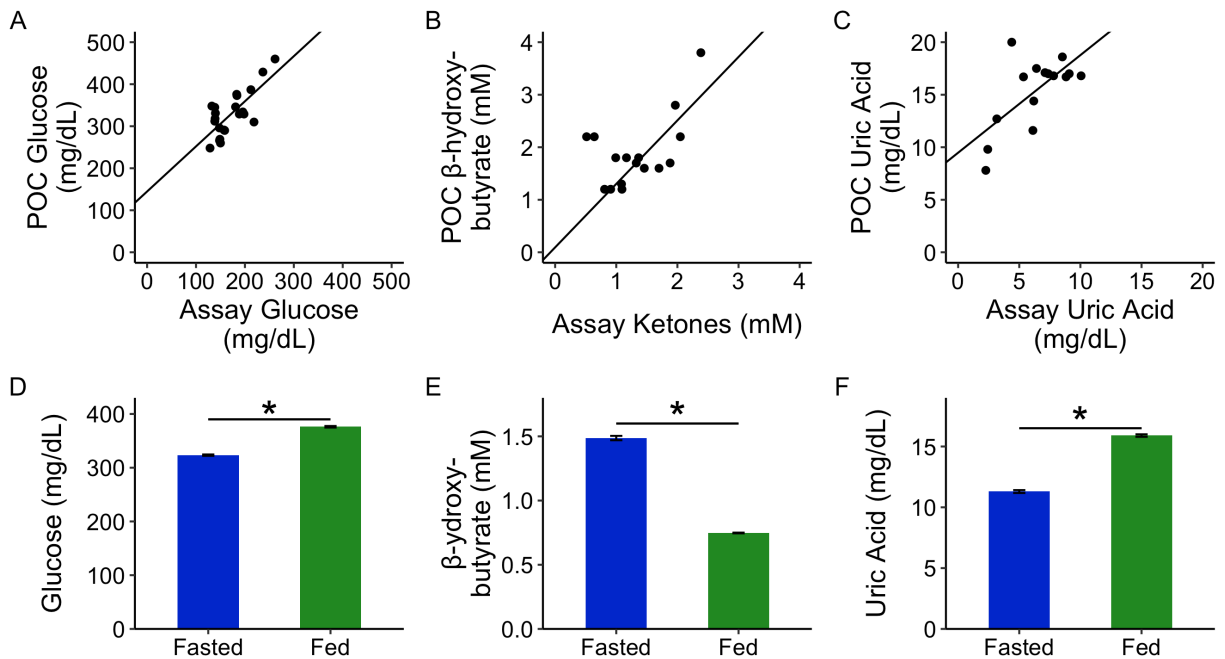


Figure 2. Validation of point-of-care devices for blood glucose, β -hydroxybutyrate, and uric acid in house sparrows. (A-C) The same blood samples were used to quantify glucose ($n=22$), β -hydroxybutyrate ($n=16$), and uric acid ($n=15$) using a standard lab assay and a point-of-care device. (D-F) In a separate experiment, house sparrows ($n=38$) were fasted overnight and the next morning, a fasted blood sample was taken 30 minutes before food addition, followed by a fed blood sample 1 hour after food addition. Bar graphs represent mean \pm SE. * indicates $p < 0.0001$. POC = point-of-care.

timinate values compared to the bench assays for glucose and uric acid, because our study only required observing changes relative to baseline concentrations, the point-of-care devices were deemed usable. Additionally, each point-of-care device was able to detect a difference between fed and fasted birds, where we would expect differences to be.

Weight

The birds' weight dropped significantly within the first 3 days of chronic stress and remained at approximately 95% of their original weight through the three weeks of chronic stress ($F_{11,125} = 4.03$, $p < 0.0001$; Figure 3). Their weight also changed significantly from the last timepoint of chronic stress through the two weeks after chronic stress, but never fully recovered ($F_{5,35} = 2.72$, $p = 0.03$).

Metabolites

Both day and state (fasted/fed) significantly affected blood glucose levels (main effect of day, $F_{1,280} = 28.52$, $p < 0.0001$; main effect of state, $F_{1,278} = 14.55$, $p < 0.0002$, day:state interaction, $F_{1,278} = 0.47$, $p = 0.49$; Figure 4A). Fasted blood glucose was significantly elevated by day

9 of chronic stress and this elevation persisted through the chronic stress period ($F_{11,121} = 5.45$, $p < 0.0001$) and did not recover once chronic stress ended ($F_{5,36} = 1.03$, $p = 0.41$). Fed blood glucose was significantly elevated by day 3 of chronic stress and the increase lasted for the duration of the chronic stress period ($F_{11,122} = 4.78$, $p < 0.0001$) and did not change after chronic stress ended ($F_{5,38} = 0.60$, $p = 0.70$).

There was a significant effect of both day and state on blood β -hydroxybutyrate levels (main effect of day, $F_{1,290} = 5.28$, $p = 0.02$; main effect of state, $F_{1,278} = 36.50$, $p < 0.0001$; day:state interaction, $F_{1,278} = 2.10$, $p = 0.15$; Figure 4B). In a fasted state, blood β -hydroxybutyrate was significantly elevated from day 3 to the end of the chronic stress period ($F_{11,122} = 5.60$, $p < 0.0001$) and this elevation persisted through the two weeks after chronic stress ($F_{5,38} = 2.22$, $p = 0.07$). In a fed state, day significantly affected blood ketone levels, however there was not a significant increase from baseline values until day 32 ($F_{11,124} = 2.06$, $p = 0.03$). There was no effect of day throughout the post-chronic stress period ($F_{5,41} = 1.99$, $p = 0.10$).

There was not a significant effect of day on blood uric acid levels ($F_{1,285} = 0.58$, $p = 0.44$), however the effect of state and the interaction between day and state were significant (main effect of state, $F_{1,278} = 21.73$, $p < 0.0001$;

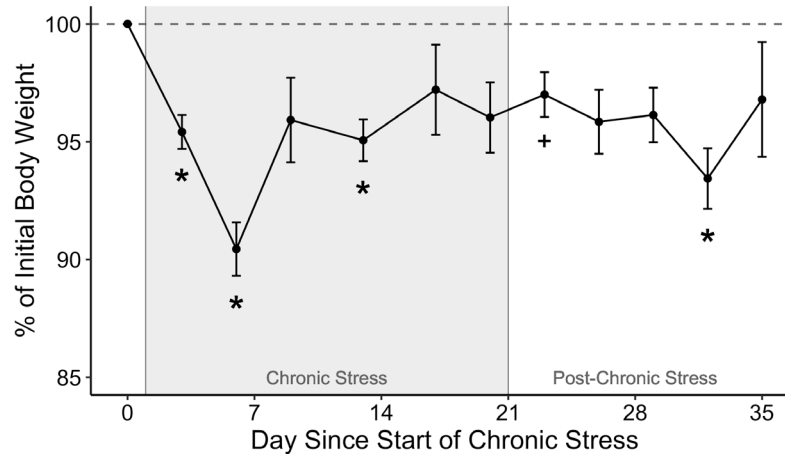


Figure 3. Body weight decreases and then stabilizes through three weeks of chronic stress (gray area) and two weeks post-chronic stress, with food restriction throughout. Graphed values represent mean percent change (\pm SE) from the starting weight taken on day zero, but statistics were performed on raw data. For days 0-21, $n=16$, for days 22-35, $n=8$. * indicates $p < 0.05$ compared to initial weight, + indicates $p < 0.05$ compared to day 20.

day:state interaction, $F_{1,278} = 7.09$, $p < 0.01$; Figure 4C). In fasted birds, uric acid did not change with day ($F_{1,122} = 1.26$, $p = 0.26$). In fed birds, blood uric acid levels were significantly elevated by day 3 of chronic stress, and this elevation persisted through the chronic stress period ($F_{1,122} = 7.48$, $p = 0.0001$). Day significantly affected uric acid levels in the post-chronic stress period ($F_{5,28} = 4.14$, $p = 0.004$), but despite an initial decrease to levels that were not significantly different from baseline, the levels then rose again to be significantly elevated. None of the post-chronic stress uric acid levels were significantly different from the last timepoint of chronic stress.

Corticosterone

Whether or not the bird was fasted (state) significantly affected corticosterone levels ($F_{1,47} = 8.56$, $p = 0.005$; Figure 5), however day and the interaction between day and state did not (main effect of day, $F_{1,121} = 1.20$, $p = 0.28$; day:state interaction, $F_{1,122} = 3.42$, $p = 0.07$). In both a fasted ($F_{6,38} = 1.88$, $p = 0.11$) and fed ($F_{11,69} = 1.25$, $p = 0.27$) state, there was no effect of day. Because corticosterone measurements require more blood volume and sample size decreased to 8 after the chronic stress period, samples for corticosterone were only collected from fed birds during the post-chronic stress period.

Behavior

Birds hopped more when a foreign object was present compared to control trials (Figure 6A; $F_{1,166} = 6.58$, $p = 0.01$), but neither day of experiment ($F_{1,174} = 0.2$, $p = 0.66$) nor the day:group interaction ($F_{1,166} = 0.29$, $p = 0.59$) affected hopping. During control trials ($F_{4,46} = 0.98$, $p = 0.43$) and object trials ($F_{9,99} = 0.76$, $p = 0.66$), there

was no effect of day.

Group (object/no object) did not significantly affect the time it took for the birds to approach their food dish ($F_{1,165} = 2.29$, $p = 0.13$), however day and the interaction between day and group did (main effect of day, $F_{1,169} = 6.22$, $p = 0.01$; day:group interaction, $F_{1,165} = 9.19$, $p < 0.005$; Figure 6B). When there was no object present, time to feed significantly decreased with chronic stress ($F_{4,46} = 8.71$, $p < 0.0001$), but did not change once chronic stress ended ($F_{2,14} = 2.78$, $p = 0.09$). Note, however, that latency to feed was quite low for all birds whenever there was no object, resulting in low variability between birds. In contrast, when a novel object was presented on the food dish, day significantly affected time to feed ($F_{9,97} = 3.82$, $p < 0.0005$) by initially decreasing until day 8, and then steadily increasing through the end of the chronic stress period and the post-chronic stress period ($F_{4,33} = 3.47$, $p = .02$).

DISCUSSION

In this study, we aimed to characterize changes in energy usage, corticosterone, and behavior through three weeks of combined chronic stress and overnight fasting, followed by two weeks of overnight fasting only. Previous avian metabolite studies have focused on migratory birds fasting during migratory bouts (reviewed in [42]) and penguins fasting during egg incubation (reviewed in [43-45]). These fasts comprise natural life-history events in the lives of those species. In contrast, studying the response to involuntary fasting in a species that does not typically fast, such as the house sparrow, is more likely to reflect a stress response that can induce wear-and-tear. Furthermore, studying species where fasting is not a nor-

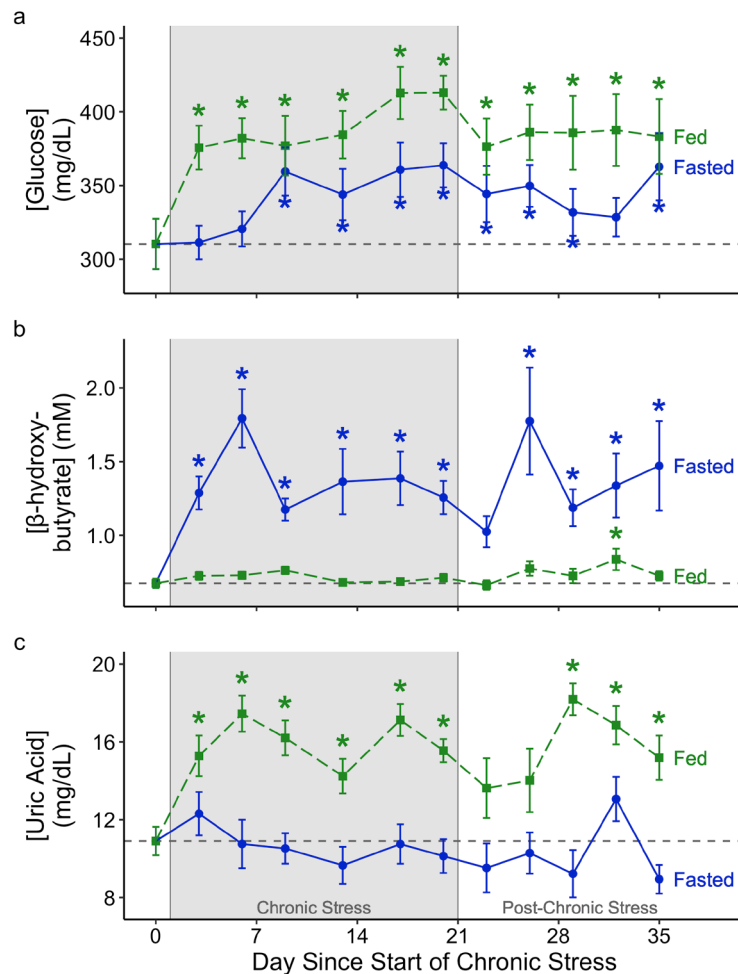


Figure 4. Plasma metabolites tracked through three weeks of chronic stress (gray area) and two weeks post-chronic stress. Glucose was measured as a proxy of carbohydrate breakdown (A), β -hydroxybutyrate was measured as a proxy of fat breakdown (B), and uric acid was measured as a proxy of protein breakdown (C). For days 0-21, $n=16$, for days 22-35, $n=8$. * indicates $p < 0.05$ compared to the initial day of sampling.

mal life-history event is relevant for understanding the impacts of increases in extreme weather events caused by climate change, which could cause more frequent food shortages. House sparrows cannot typically fast longer than 24-32 hours [6,7], so it is possible to push them close to their physiological limits in a short amount of time.

Weight

Weight loss is a hallmark of chronic stress and the weight lost by the birds in the present study (Figure 3) is consistent with other studies on chronic stress in house sparrows [39,46], starlings [9,47], and white-crowned sparrows [48]. However, unlike both starling studies, which also involved a similar chronic stress protocol with recovery period, the house sparrows in this study did not exhibit a rebound in their body weight when chronic stress ended (Figure 3). This difference could be species

specific, as one other house sparrow study did not exhibit a dramatic weight gain after the end of chronic stress [30]. When food was restricted predictably and unpredictably, starlings increased body weight in both conditions, suggesting that food restriction itself was not a stressor [4], and thus not affecting weight in the post-chronic stress period. However, the decrease in body weight in the current study supports the conclusion that the protocol successfully induced chronic stress.

Metabolites

The birds in the present study exhibited an increase in blood glucose in both a fed and fasted state throughout three weeks of chronic stress that did not recover within two weeks after chronic stress (Figure 4A). At the time of the fasted blood sample, the birds had not had access to their food for 18 hours, so it is not surprising to see a sig-

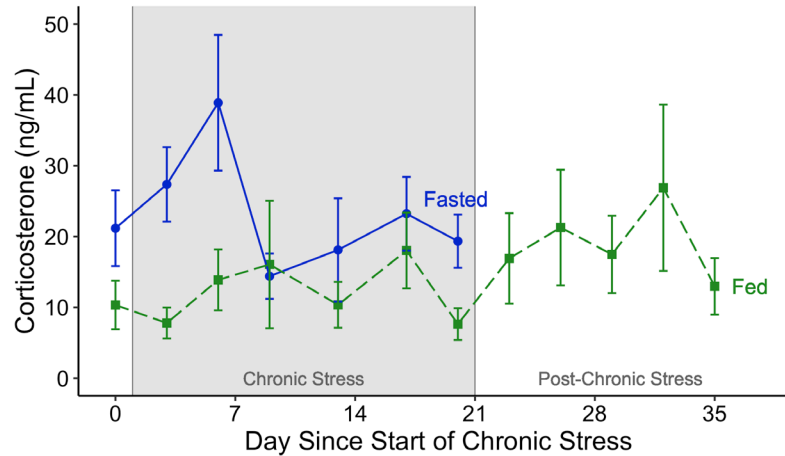


Figure 5. Blood corticosterone through three weeks of chronic stress (gray area) and two weeks following.

Fasted samples were taken from half of the birds ($n=8$) and fed samples were taken from the other half ($n=8$).

We were unable to measure corticosterone in fasted birds during the post-chronic stress phase. Graphed values represent mean \pm SE. A linear mixed effect model showed a significant difference between states (fasted/fed), but no significant changes throughout days of the experiment.

nificantly higher concentration of glucose in the blood 60 min after the birds are given their food. This suggests that they ate as soon as food was available (which is also supported by the low latency to feed when food was presented without novel objects; Figure 6; [49]). However, it is interesting that throughout the five-week experiment, the fasted and fed blood samples increased in parallel (with a one-week lag), a pattern likely induced by the chronic stress protocol. The elevated blood glucose could reflect an increase in the amount of food eaten by the birds, however, if this were the case, we might expect to see an increase in weight (Figure 3) or in fed uric acid levels (Figure 4C), but neither of those variables increased in concert with glucose. Similarly, corticosterone can induce an increase in glucose [50,51], but in the present experiment, corticosterone (Figure 5) did not increase in parallel with glucose, suggesting that another mechanism was involved in the regulation of glucose during chronic stress. Other chronic stress studies [29,52-54] did not report changes in glucose. Food restriction combined with chronic stress can have an additive effect [55] and glucose is very tightly regulated in birds [14,56,57], so it is possible that we were able to induce greater allostatic load in the birds than previous studies with the combination of fasting and chronic stress.

Fat stores are the primary source of energy in the second phase of fasting [5,6,58]. When carbohydrate stores have been depleted, fat stores are converted to usable ketones (such as β -hydroxybutyrate) via lipolysis and oxidation [15]. Thus, it is clear from the 200% increase of blood β -hydroxybutyrate at 18 hours of fasting (Figure 4B) that birds were in the second phase of fasting. This elevation was consistent for the entire five-week exper-

iment, indicating that the amount of fat that needed to be broken down remained fairly constant. It is also clear that as soon as food was available, the birds immediately switched to using energy from their diet, as opposed to continuing to tap into their fat stores. The levels of β -hydroxybutyrate in a fed state maintained at about 100% of baseline levels. We are unaware of any research on changes in ketones with chronic stress in birds, but fat scoring as an indirect measure of fat metabolism with chronic stress [59], as well as short-term fasting [21] also suggests fat utilization.

Uric acid is the primary nitrogenous waste in birds and is produced when protein is catabolized [17]. Uric acid was therefore used in the current study as a proxy of protein breakdown, which is a hallmark of the third phase of fasting [5,14,57]. In a fasted state in the current study, there was no change in uric acid levels (Figure 4C), which suggests that the birds never tapped into their protein reserves during the 18-hour fast, and thus did not enter the third phase of fasting. The dramatic increase of plasma uric acid observed when the birds were in a fed state (Figure 4C) is not because they were tapping into their protein reserves when they were satiated, but because a large component of the birds' diet was sunflower seeds, which are very high in uric acid [8]. We include these data for completeness, but these levels do not reflect the underlying physiology of endogenous protein metabolism.

Khalilieh et al. [6] argued that house sparrows cannot fast for a long time because of their inability to sequentially utilize energy stores, based on a combination of plasma metabolite analyses and $^{13}\text{CO}_2$ breath-testing. However, their measures of blood glucose and ketones appear to

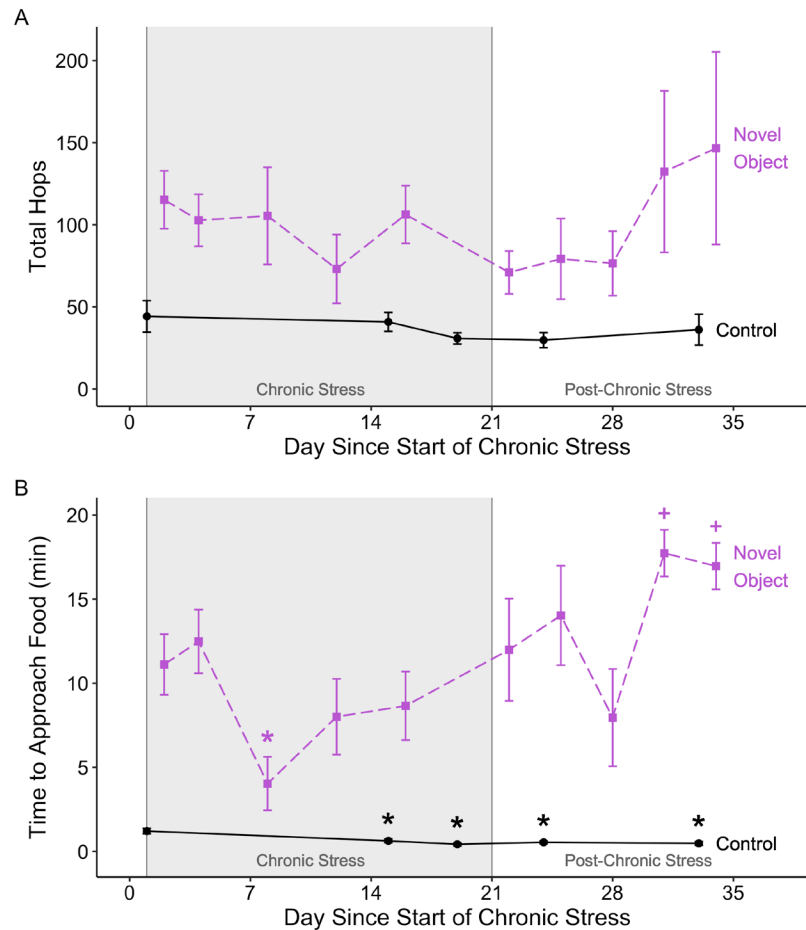


Figure 6. Behavioral changes through chronic stress. Total activity (**A**) and time to approach food (**B**) when a novel object is or is not present during three weeks of chronic stress (gray area) and two weeks post-chronic stress. For days 0-21, $n=16$, for days 22-35, $n=8$. * indicates $p < 0.05$ compared to initial latency, + indicates $p < 0.05$ compared to day 16.

show a well-defined phase II and a slightly less well-defined phase I [6]. This finding aligns well with our results: β -hydroxybutyrate increased dramatically at 18 hours of fasting compared to glucose and uric acid (Figure 4). Additionally, although low-to-mid levels of glucose are to be expected in phase II, glucose increased in both fed and fasted states throughout the five-week experiment (Figure 4A), potentially indicating that the birds needed more energy from carbohydrates during phase II to cope with the chronic stress. Despite the fact that wear-and-tear did not manifest in a premature shift from phase II to phase III, the glucose data support the idea that chronic stress resulted in wear-and-tear and made it more likely that the birds would enter homeostatic overload during the fast. The birds were having to rely upon energy sources other than diet to sustain the elevated fasting glucose levels, which reflects a decreased reactive scope to cope with the fast.

Corticosterone

Despite an initial slight spike from days 3-8, plasma corticosterone levels did not change as the experiment progressed in either the fed or the fasted group (Figure 5). This result is not necessarily surprising, because although the corticosterone response to acute stress is quite robust, responses to chronic stress are variable (reviewed in [60]). Previous studies in house sparrows are similarly variable, with some showing no change [10], increases [46], or decreases [61]. Though corticosterone is known to mediate the transition between phases of fasting [12,13], food restriction itself can be perceived as a stressor. Although fasting can lead to an increase in corticosterone in both wild [62] and experimental [21] settings, this response is not universal because other experimental studies report no change in corticosterone [4]. Although it is possible that our birds did not perceive the food restriction as a stressor, it is likely that they were experiencing chronic

stress because they lost weight (Figure 3) [47].

Behavior

Behavior is an integrated response to internal and external processes [63]. Throughout the experiment, the latency to approach food without an object present decreased slightly, but significantly (Figure 6B), while overall activity did not change (Figure 6A). This finding is similar to previous studies that also show a significant decrease in approach time (but not total activity) throughout a chronic stress period when an object is not present [38,64]. On day 8, approach latency to the novel object decreases substantially (Figure 6B, third point in “novel object” line), but total activity does not (Figure 6A, third point in “novel object” line). These results also align with previous studies suggesting that the decrease in neophobia during chronic stress reflects the birds’ willingness to take more risks to obtain food during a stressful state [65,66]. However, it is unclear why this response is transient in the present study.

In contrast to the chronic stress period, the approach latency (Figure 6B) during the post-chronic stress period increased significantly and total activity increased slightly (Figure 6A) from chronic stress levels in the object trials. Interestingly, the two days with significantly higher approach latencies coincided with the same two days with considerably larger variations in total activity. In other words, novel objects appeared to elicit a stronger response once the chronic stress, but not the fasts, had ended and total activity becomes more variable among birds. If chronic stress was driving the decrease in neophobic behavior (this study, [65,66]), the increase during object trials seen in the post-chronic stress period could be reflecting a recovery of behavior that is on a different timescale than recovery of the physiological variables measured [38].

CONCLUSIONS

Three weeks of chronic stress and an extended daily fast appeared to result in wear-and-tear as evidenced by an increase in plasma glucose in both a fed and fasted state. The increase in glucose during the chronic stress period did not recover within two weeks after chronic stress ended, indicating that the effects of wear-and-tear are not short lived. House sparrows also appear to be dependent on fat reserves after 18 hours without food, consistent with models of phase II of fasting. However, they stop tapping into their fat reserves as soon as food is available again. Because protein usage did not change, the birds did not appear to enter phase III of fasting during chronic stress, but it is possible that with a longer period of chronic stress, they might have. Alternatively, there can be long-lasting effects of fasting that can make

animals more resistant to further fasts [67]. Whether this occurred in the present study is not known. Additionally, the period of chronic stress appeared to have an effect on behavior when a novel object was and was not present, though not in the same manner. Lastly, although there were clear effects of chronic stress on the variables measured, corticosterone did not significantly change from pre-chronic stress levels, suggesting that corticosterone was not driving the changes we measured.

The final conclusion is that the combination of an extended fast and chronic stress created wear-and-tear but did not push the birds into homeostatic overload. There were seemingly minor changes in corticosterone and behavioral regulation, and chronic stress did not alter the fed vs fasted transitions in fat and protein metabolism. Only glucose mobilization seems to have been affected. Consequently, layering the wear-and-tear associated with chronic stress did not result in a quicker transition to phase III of fasting. It is still possible that chronic stress could accelerate the transition to phase III, but either the fast was not long enough or the *ad libitum* daily feeding was sufficient to prevent that transition. Regardless, house sparrows appear to be robust to resisting the combined effects of chronic stress and fasting.

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