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Responses of New World flying squirrels to the acute stress of capture and handling

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Northern (*Glaucomys sabrinus*) and southern (*G. volans*) flying squirrels have glucocorticoid (GC; stress hormone) levels higher than most vertebrates but virtually no binding capacity for these GCs via the carrier protein, corticosteroid-binding globulin. Thus, their total GCs are essentially all free and biologically active. However, the GC estimates come from blood samples taken after squirrels had been in live traps, and thus in a stress-induced state. Obtaining baseline values for physiological variables is valuable for assessing the response of vertebrates to stressors in their environment. We compared baseline plasma total cortisol levels (within 3 min of capture) to stress-induced levels (after 30 min of trap restraint) in both flying squirrel species. We recorded baseline cortisol levels that were some of the highest ever reported for mammals, indicating their stress axes operate at a higher set point than most other species. As part of the stress response, we also measured 4 indices in addition to cortisol. Total cortisol and free fatty acids increased in both species, as predicted. In contrast with our predictions, blood glucose and neutrophil/lymphocyte ratio showed no overall change, and hematocrit decreased significantly. New World flying squirrels therefore appear to have a stress response that differs from many other mammals. The selective forces driving the physiology of these animals remain elusive, but this lineage may provide an interesting comparative system for the study of stress axis function and its evolution among vertebrates.

Key words: cortisol, energy mobilization, field endocrinology, hematocrit, immune function, mammals

New World flying squirrels, the northern (*Glaucomys sabrinus*) and the southern (*G. volans*) flying squirrel, have glucocorticoid (GC) levels that are among the highest of all vertebrates (Desantis et al. 2013). GCs are stress hormones (cortisol being the main GC in flying squirrels) that are produced in the adrenal cortex as a result of the neuroendocrine pathways involved in the hypothalamic–pituitary–adrenal (HPA) axis or, more simply, the stress axis (Sapolsky et al. 2000). The high cortisol levels in flying squirrels are coupled with a virtually nonexistent binding by corticosteroid-binding globulin (CBG), that in most other mammals binds GCs tightly and permits only 5–10% to be unbound (i.e., free) and biologically active (Perogamvros et al. 2012; Breuner et al. 2013; Desantis et al. 2013). This protective carrier protein therefore normally acts as a buffer to the tissues against potent GCs and is a reservoir for them in the blood (Westphal 1983). In flying squirrels, however, most (90–95%) circulating cortisol is in the free state (Desantis et al. 2013), where it can potentially pass through cell membranes and activate tissue receptors that invoke the downstream effects of GCs associated with a response to stress (Mendel 1989, 1992).

The stress axis is highly conserved across the vertebrates, but New World flying squirrels have adopted a strategy that stands in marked contrast to most others, and thus we wished to explore how their responses to acute stressors compare with those of other mammals. Previous conclusions about their stress axes were based on an analysis of stress-induced blood plasma samples collected during a regular field protocol where live traps were set and then checked 2–3 h later (Desantis et al. 2013). To properly assess the stress physiology of any mammal, it is critical to first evaluate how their HPA axis responds to acute stress by comparing baseline levels of GCs and related hematological variables with stress-induced levels (Reeder

and Kramer 2005; Delehanty and Boonstra 2009, 2012). Such "stress profiles" have been assessed in relatively few mammals, likely due to difficulties associated with capture (reviewed by Reeder and Kramer 2005; but see Place and Kenagy 2000). The HPA axis normally responds within 3–5 min of a stressor being perceived, resulting in an increased secretion of GCs, and thus baseline blood samples in wild animals must be and have been collected within 3 min of capture (e.g., Boonstra et al. 1998; Place and Kenagy 2000; Reeder et al. 2004a; Romero et al. 2008). To collect paired, stress-induced samples, the same individuals are subjected to a moderate stressor for a short time; 30 min is a commonly used time period in such studies (e.g., Place and Kenagy 2000; Romero et al. 2008).

We define a stress profile as a suite of physiological variables that describe both the baseline state of HPA axis functioning and the effect its activation has on GC targets. Because approximately 10% of the genome is influenced by GCs, with targets including genes controlling metabolism, growth, repair, reproduction, and resource allocation (Le et al. 2005), we surveyed a variety of functional responses. Our direct measure of a stress response by the HPA axis was total plasma cortisol concentration. Meanwhile, our measurements of GC targets include those involved with energy mobilization (free fatty acid [FFA] and blood glucose levels), an indicator of general health (hematocrit; the percentage of blood volume comprised of red blood cells), and immune function (changes in proportions of leukocytes, particularly the neutrophil-to-lymphocyte [N:L] ratio).

We evaluated stress profiles for northern and southern flying squirrels and made comparisons with other mammals, the majority of which have ~ 90% of GCs tightly bound to CBG (Desantis et al. 2013). We predicted that responses would be similar in direction and magnitude between these 2 sister species, but that some aspects of flying squirrel stress profiles might differ from other mammals, as part of their unusual stress physiology. From studies using similar methodology (i.e., where baseline and 30- to 60-min stress-induced samples were taken, and acute stressors were trapping or handling related), we predicted that cortisol (e.g., Place and Kenagy 2000), FFAs (e.g., Handasyde et al. 2003), blood glucose (e.g., Fletcher and Boonstra 2006), and the N:L ratio (e.g., Davis et al. 2008) would increase, and that hematocrit would either increase (e.g., Boonstra et al. 2001) or show no change because this variable has been shown to change only over longer time periods (e.g., Clinchy et al. 2004; Delehanty and Boonstra 2009).

Materials and Methods

Study species.—Flying squirrels are nocturnal, arboreal rodents that are active year-round. Northern flying squirrels inhabit the mixed-deciduous and coniferous forests across Canada, Alaska, and the northeastern border states of the United States and areas of high elevation extending south throughout the Appalachian, Rocky, Cascade, Sierra Nevada, and Pacific Coastal mountains. Southern flying squirrels inhabit the hardwood deciduous forests of southeastern Canada and the eastern United States and areas of high elevation in Mexico and Central America. Both species occur in a small area of sympatry in the mixed-deciduous forests of eastern Canada and the northeastern United States (Dolan and Carter 1977; Wells-Gosling and Heaney 1984; Thorington et al. 2012).

The main food sources for northern flying squirrels are lichens and hypogeous fungi associated with several tree types (Weigl 1978; Loeb et al. 2000; Dubay et al. 2008) but are probably consumed more often when other seeds, fruits, and insects in their omnivorous diet are limited or unavailable (Vernes et al. 2004). Whether this species caches food extensively is not well documented, but they are thought to do so much less than southern flying squirrels as they have often been observed foraging for food under the snow in winter (Wells-Gosling and Heaney 1984). Southern flying squirrels are also omnivorous, but rely more heavily on nuts and seeds from masting hardwood trees (Dolan and Carter 1977; Weigl 1978). Southern flying squirrels are scatter hoarders (Winterrowd 2008) and known to cache food for winter with this behavior peaking around November (Dolan and Carter 1977). Although they are seasonally gregarious, nesting in groups at this time (Garroway et al. 2013), individuals appear to maintain solitary food caches (Winterrowd 2008; Murrant et al. 2014). Social nesting by northern flying squirrels has been documented much less often, and with smaller group sizes (Weigl 1974; Cotton and Parker 2000; Hough and Dieter 2009), but females of both species become solitary during breeding (Layne and Raymond 1994).

Study sites.—The principal study site was located near Mississagua Lake in the Kawartha Lakes Region of south-central Ontario, Canada (44°41′18″N/78°20′8″W; 10 southern and 1 northern flying squirrel[s] caught here). Livetrapping was conducted in a portion of contiguous forest just west of Kawartha Highlands Provincial Park on the southern edge of the Canadian Shield (the transition zone between the Carolinian forests of southern Ontario and the Boreal forests of northern Ontario). Two additional sites were located in south-central Ontario, 70–120 km from the primary site: one at Riley Lake near the town of Gravenhurst (44°50′23″N, 79°12′13″W; 4 southern and 1 northern flying squirrel[s]) and another in the Trent Nature Areas in Peterborough, Ontario (44°21′27″N, 78°17′46″W; 1 northern flying squirrel). A 4th site was in boreal forest and located approximately 16 km southwest of Lake Abitibi in northern Ontario, on the southeastern edge of Munro Lake (48°34′31″N, 80°8′3″W; 4 northern flying squirrels).

Livetrapping and blood sampling.—Blood sampling took place between mid-September and mid-November in the fall of 2009 and 2011. We chose this time of year because it was outside of the breeding season and thus minimized the likelihood of reproductive hormones and related cycles of stress hormones interfering with the interpretation of our results. We sampled 21 squirrels: 14 southern and 7 northern flying squirrels. Sample sizes differed among measured variables owing to low blood volumes obtained for some animals (Table 1). All animals were in nonreproductive condition (males' testes were abdominal and females were neither pregnant nor lactating). The 2 species are easily distinguishable in this area based on pelage, visual appearance of overall body size, and body weights (weight

Table 1.—Sample sizes for males and females within each species for all measured response variables. Total *n* denotes the pooled sample sizes used for statistical analyses. Sample sizes differ among variables due to low blood volumes obtained from some animals. FFA = free fatty acids; NFS = northern flying squirrel (*Glaucomys sabrinus*); N:L = neutrophil-to-lymphocyte ratio; SFS = southern flying squirrel (*G. volans*).

Response variable	Species	Sex	*n*	Total *n*
Total cortisol, glucose	SFS	M	5	
		F	9	14
	NFS	M	4	
		F	3	7
FFA	SFS	M	1	
		F	3	4
	NFS	M	1	
		F	2	3
Hematocrit	SFS	M	3	
		F	5	8
	NFS	M	3	
		F	2	5
All leukocytes, N:L	SFS	M	4	
		F	6	10
	NFS	M	3	
		F	1	4

ranges do not overlap). Southern flying squirrels were identified by the presence of pure white ventral fur, and body weights ranged from 59 to 78 g ($\overline{X}$ ± *SE*, 65.8 ± 1.4 g), consistent with descriptions given in Dolan and Carter (1977). Northern flying squirrels were identified by the presence of ventral fur with dark grey at the base and white at the tips, and body weights ranged from 108 to 132 g ($\overline{X}$ ± *SE*, 118.1 ± 3.5 g), consistent with descriptions given in Wells-Gosling and Heaney (1984) and Banfield (1987).

Males and females within each species were pooled because previous data (Desantis et al. 2013) show that total cortisol levels in the nonbreeding season do not differ between the sexes (southern flying squirrels: $t_4 = 0.75$, $P = 0.49$; northern flying squirrels: $t_{12} = 0.41$, $P = 0.69$). Research on live animals followed American Society of Mammalogists' guidelines (Sikes et al. 2011), and all procedures were performed in accordance with the guidelines of the Canadian Council on Animal Care and were approved by the Trent University Animal Care Committee (#22196).

To obtain blood samples within 3 min of capture, 3–5 tomahawk live traps (Model 102; Tomahawk Live Trap Company, Hazelhurst, Wisconsin) were baited each night with peanut butter and fastened to wooden platforms mounted on trees surrounding an open area. These were approximately 10 m from where we waited; near enough for us to hear the traps close, but far enough that the animals would not be afraid to enter. Traps were set just prior to dusk and monitored continuously for 3–5 h. Timing began as soon as a trap was triggered. Animals were removed and placed in a cloth handling bag with mesh fabric at 1 end (this portion of the procedure usually took 30–40 s). Isoflurane was administered via the nose cone method (a glass tube containing isoflurane-soaked cotton), and a baseline blood sample was then drawn via the suborbital sinus using a heparinized Pasteur pipette. The average time (± *SE*) to collect the baseline blood sample was 2:27 ± 0:05 min:s, and all were collected in under 3 min (range: 1:27 to 2:56). It has been shown in some species of birds however that GCs can begin to increase significantly after only 2 min (Romero and Reed 2005), and thus we assessed our baseline cortisol values against time to collect these blood samples. There was no significant relationship for either species (southern flying squirrels: $F_{1,12} = 1.56$, $P = 0.24$; northern flying squirrels: $F_{1,5} = 0.03$, $P = 0.87$), which indicates that cortisol levels did not begin to rise until sometime after 3 min.

Following collection of the baseline sample, animals were returned to the trap and the trap was placed inside a pillowcase for 30 min. This acted as a stressor to simulate an animal being held in a live trap during a regular trapping protocol, where traps are set and then checked hours later. After approximately 30 min of acute stress (which included the stress of handling to obtain blood samples), squirrels were placed back in the handling bag and anesthetized. A second suborbital sinus blood sample was then drawn, and this we term the stress-induced sample. The average time (± *SE*) to collect the stress-induced sample after capture was 35:23 ± 0:52 (range: 30:00 to 40:00). For 1 individual southern flying squirrel, the stress-induced sample was not collected until 50 min postcapture because of delays in processing due to a surplus of squirrels on this particular night. Values for this individual were within the range of other individuals, so data for the squirrel were retained for all analyses.

Each blood sample was 200–300 µl in volume. Glucose levels (mg/dl) were obtained immediately using residual blood from each sample collection pipette (FreeStyle Freedom Lite; Abbott Diabetes Care Inc., Alameda, California). Samples were stored in 0.6-ml Eppendorf vials and kept on ice until return to the laboratory at Trent University approximately 3–4 h later.

Laboratory analyses: hematology.—Upon return to the laboratory each night, sample vials were gently inverted to re-homogenize whole blood samples. Blood was then drawn into heparinized microhematocrit capillary tubes in duplicate (Fisherbrand; Fisher Scientific, Pittsburgh, Pennsylvania), spun in a microhematocrit centrifuge (IEC Micro-MB, Model 120; Thermo Electron Corporation, Milford, Massachusetts), and hematocrit was measured. Blood smears were created in duplicate on glass slides for leukocyte differentiation (i.e., a leukogram) and stained using a Three-Step Stain Set (Thermo Fisher Scientific, Kalamazoo, Michigan). Proportions of each cell type (monocytes, eosinophils, basophils, neutrophils, and lymphocytes) out of a total of 100 leukocytes were recorded by a single individual, and the N:L ratio was calculated for each sample. Source blood samples were spun in an Eppendorf mini centrifuge at 15,700 × *g* (Model 5415 D; Hamburg, Germany) and plasma was removed for storage at −80°C until use in the following procedures.

Laboratory analyses: measurement of plasma total cortisol.—Total cortisol was measured using a commercially available radioimmunoassay (Clinical Assays GammaCoat Cortisol ^{125}I RIA Kit; DiaSorin, Stillwater, Minnesota). The kit was

validated for parallelism on flying squirrel plasma. Tests for differences between slopes on $\log_{10}$-transformed data showed that serially diluted plasma curves for both species were parallel to the assay standard curve (southern flying squirrels: $F_{1,11} = 0.40$, $P = 0.54$; northern flying squirrels: $F_{1,11} = 0.75$, $P = 0.41$). Linearity of the standard curve was tested beyond the standards provided in the kit (0–600 ng/ml) up to 3,500 ng/ml because of the high cortisol concentrations measured in flying squirrels. The intra- and interassay coefficients of variation (*CV*) were 2.9% and 9.5%, respectively, and the minimal detectable dose of the assay was 2.1 ng/ml. Although in most species CBG levels should be measured, so that free (i.e., biologically active) GC concentrations can be calculated (Breuner et al. 2013), flying squirrels have virtually no binding capacity (90–95% of their cortisol is free—Desantis et al. 2013). Hence, we report only total cortisol.

Laboratory analyses: measurement of plasma FFAs.—We measured FFAs using a NEFA-C kit (Wako Chemicals USA Inc., Richmond, Virginia) modified to be used with a 96-well plate (Johnson and Peters 1993; Delehanty and Boonstra 2009). Plasma was not diluted prior to the assay. The intraassay *CV* was 6.3%, and since all samples were run within a single assay, there was no interassay *CV*. The minimal detectable dose of the assay was 0.0014 mM, and linearity ranged from 0.10 to 4.00 mM.

Statistical analyses.—All data are expressed as means ± *SE* unless otherwise stated and were analyzed using GraphPad Prism version 5.00 (GraphPad Prism 2008). Two-way repeated-measures analysis of variance (ANOVA) was used to test for effect of sampling time (baseline versus stress-induced samples) and of species (northern versus southern flying squirrels), and the Bonferroni post hoc test was used to determine where means differed significantly from one another for all variables unless otherwise stated below ($P \leq 0.05$). We assessed normality using the D'Agostino & Pearson omnibus K2 test, and homoscedasticity using Bartlett's test for equal variances. Within-subject variability was too high for FFAs ($F_{1,5} = 2.09$, $P = 0.22$), and too low for basophils ($F_{1,12} = 2.09$, $P = 0.11$) relative to the variation explained by the main effects for an effective paired design, and thus we report the results from a 2-way ANOVA for these variables. For glucose, pairing was ineffective ($F_{1,19} = 1.66$, $P = 0.14$) and variances were not equal. Transforming the data did not equalize variances and thus we analyzed the glucose data separately for the 2 species using an unpaired *t*-test with Welch's correction, which does not assume equal variance.

Results

The 2-way repeated-measures ANOVA for changes in total cortisol levels indicated an effect of sampling time ($F_{1,19} = 23.30$, $P = 0.0001$; Fig. 1a) and species ($F_{1,19} = 18.76$, $P = 0.0004$), but no interaction effect ($F_{1,19} = 0.17$, $P = 0.69$). Cortisol increased 37.7% in southern flying squirrels from a baseline of 1,663 (± 188.4) ng/ml to a stress-induced level of 2,290 (± 179.9) ng/ml. In northern flying squirrels, cortisol increased 84.0% from a baseline of 628.7 (± 144.1) ng/ml to a stress-induced level of 1,157 (± 99.29) ng/ml. Thus on average, southern flying squirrels had 2.6 times the baseline cortisol levels of northern flying squirrels, and 2.0 times the stress-induced levels.

For changes in indicators of energy mobilization, FFAs showed an effect of sampling time ($F_{1,10} = 7.54$, $P = 0.02$; Fig. 1b) and species ($F_{1,10} = 14.56$, $P = 0.003$), but no interaction effect ($F_{1,10} = 0.006$, $P = 0.94$). FFAs increased 363.6% in southern flying squirrels from a baseline of 0.05 (± 0.03) mM to a stress-induced level of 0.23 (± 0.09) mM and increased 57.1% in northern flying squirrels from a baseline of 0.30 (± 0.06) mM to a stress-induced level of 0.47 (± 0.05) mM. Northern flying squirrels therefore had 6.0 times the baseline FFA levels of southern flying squirrels, and 2.0 times the stress-induced levels, on average. In contrast to FFA, there was no overall change in glucose with sampling time in either species (southern flying squirrels: Welch corrected $t_{17} = 1.89$, $P = 0.08$; northern flying squirrels: Welch corrected $t_{11} = 0.53$, $P = 0.61$; Fig. 1c).

Hematocrit showed an effect of sampling time ($F_{1,11} = 6.82$, $P = 0.02$; Fig. 1d), but no species ($F_{1,11} = 0.02$, $P = 0.88$) or interaction effect ($F_{1,11} = 1.53$, $P = 0.24$). Hematocrit decreased 9.9% in southern flying squirrels from a baseline of 47.8 (± 1.25) % to a stress-induced level of 43.0 (± 1.44) % and decreased 3.7% in northern flying squirrels from a baseline of 45.9 (± 2.42) % to a stress-induced level of 44.2 (± 2.36) %.

None of the 5 leukocyte types nor the N:L ratio showed an effect of sampling time or species, and there was no interaction (Table 2). For all comparisons, $P \geq 0.38$ except for species-level differences in lymphocytes ($F_{1,12} = 3.69$, $P = 0.08$) and monocytes ($F_{1,12} = 3.39$, $P = 0.09$). N:L ratios were lower than 1.00 for both species at both sampling times, where lymphocytes were the most abundant cell type (~ 60–75%), and neutrophils and monocytes each accounted for ~ 10–20% of leukocyte totals.

Discussion

In response to the capture and blood sampling protocol, northern and southern flying squirrels had stress profiles with differences between baseline and stress-induced plasma samples for some measured variables: cortisol, FFAs, and hematocrit. The direction of change was consistent with our prediction only for cortisol and FFAs, though for all variables (regardless of our prediction), the direction of change was the same for both species. Flying squirrels therefore appeared to show differences in their response to acute stress compared to other mammals and did so in a genus-specific fashion (summarized in Table 3). However, cortisol and FFA levels were species specific, suggesting differences between their stress axes may reflect or have arisen from ecological niche differences. This idea requires further study.

Total cortisol.—An increase in total cortisol concentration following 30 min of acute stress was consistent with our prediction and showed that both flying squirrel species have baseline levels that are significantly lower than their stress-induced levels (Fig. 1a); stress-induced levels in our study were similar to the nonbreeding season stress-induced levels reported by Desantis et al. (2013). While it is possible that the high

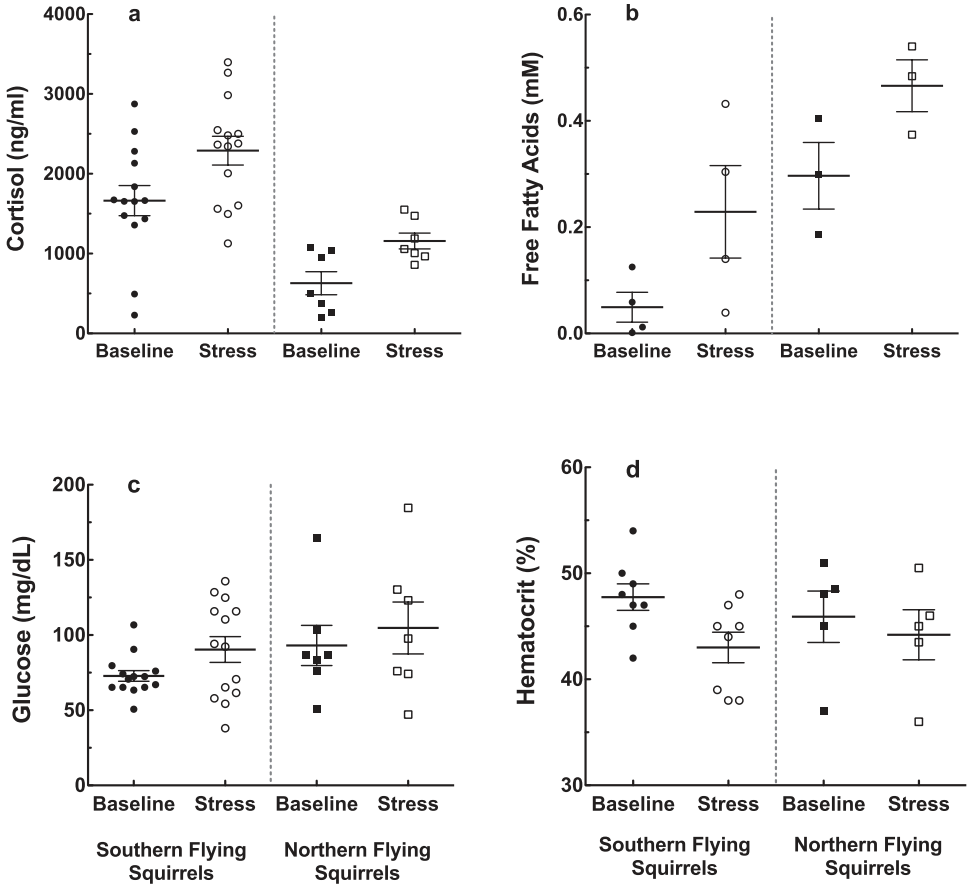

Fig. 1.—Endocrinological and hematological responses to 30 min of trap-restraint stress in southern (*Glaucomys volans*) and northern (*G. sabrinus*) flying squirrels. Baseline samples were drawn within 3 min of capture. a) Cortisol, b) free fatty acids, c) glucose, and d) hematocrit. Dark central lines are means, and surrounding bars are *SE*. Individual data points are shown by circles or squares on each graph. Sample sizes are given in Table 1.

Table 2.—Leukogram results, where the proportion of each leukocyte type was calculated out of 100 observed leukocytes in baseline and stress-induced blood samples. Data are means ± *SE*. No significant changes in proportions were found over time for any cell type nor for the N:L ratio in either species. N:L ratio = neutrophil-to-lymphocyte ratio.

Leukocyte type (%)	Southern flying squirrels (10)		Northern flying squirrels (4)	
	Baseline	Stress induced	Baseline	Stress induced
Neutrophils	13.9 ± 2.4	14.0 ± 2.1	12.25 ± 2.7	11.75 ± 2.2
Lymphocytes	62.25 ± 3.0	65.45 ± 3.4	73.38 ± 2.5	72.88 ± 2.2
N:L	0.24 ± 0.05	0.22 ± 0.04	0.17 ± 0.04	0.16 ± 0.04
Monocytes	20.75 ± 2.7	17.45 ± 3.1	11.12 ± 2.1	11.38 ± 1.8
Eosinophils	1.60 ± 0.3	1.65 ± 0.5	1.50 ± 0.5	1.88 ± 0.4
Basophils	1.50 ± 0.3	1.45 ± 0.3	1.75 ± 0.3	2.00 ± 0.6

Table 3.—Summary of results found in the current study compared with predictions based on expectations from the literature. Two of the 5 response variables showed results consistent with predictions (denoted by a check), but the remaining 3 showed unexpected results (denoted by an x). The direction of change or a result of no change over 30 min of acute stress was the same for both southern (*Glaucomys volans*) and northern (*G. sabrinus*) flying squirrels for all response variables. N:L ratio = neutrophil-to-lymphocyte ratio.

Response variable	Prediction	Result
Total cortisol	↑	✓—Increased
Free fatty acids	↑	✓—Increased
Glucose	↑	x—No change
Hematocrit	↑ or →	x—Decreased
N:L ratio	↑	x—No change

baseline cortisol levels we report are because flying squirrels have a faster response time than is typically assumed for mammals (3 min), precise evaluation of this was beyond the scope of this study. However, many of our baseline samples were collected within 2 min of capture, and our regression analysis (see "Materials and Methods") showed that there was no pattern of increase in cortisol levels as time to collect baseline samples increased towards 3 min for either species. Thus, collecting samples within 3 min of capture appeared to reflect true baseline cortisol levels in flying squirrels.

The baseline cortisol levels we detected are some of the highest ever reported (but see Reeder et al. 2004b—variable flying foxes [*Pteropus hypomelanus*] in reproductive condition show baseline GC levels similar to southern flying squirrels). By comparison, red squirrels (*Tamiasciurus hudsonicus*) were previously thought to have one of the highest total cortisol concentrations among sciurids, but the mean *baseline* concentration of northern flying squirrels is similar to the mean *stress-induced*

concentration in red squirrels (~ 735 ng/ml—Boonstra and McColl 2000). These high baseline GC levels indicate that the stress axes of New World flying squirrels operate at a higher set point than most other vertebrates.

The reasons for species-specific and genus-specific GC levels in flying squirrels are likely to do with differences in biological processes like levels of gene expression of glucocorticoid and mineralocorticoid receptors and perhaps of the carrier proteins CBG and serum albumin and tissue sensitivity (i.e., the affinity of glucocorticoid and mineralocorticoid receptors for cortisol—discussed further in Desantis et al. 2013). Other reasons could include varying levels of enzymes that convert active GCs to their nonactive forms and vice versa (e.g., 11β-HSD1 and 2), metabolic demands, and metabolism of the hormone (Chrousos et al. 1982; Scammell et al. 2001; Romero et al. 2008; Desantis 2011; Jessop et al. 2013). These differences may result from evolutionary responses to the different environments each species inhabits, or perhaps compensation for random mutations within their stress axes (Jessop et al. 2013). However, to the best of our knowledge, the physiological mechanisms and evolutionary explanations for this variation among species have been largely unexplored.

FFA and blood glucose.—The increased levels of GCs produced during a stress response stimulate gluconeogenesis in the liver, decrease uptake of glucose by peripheral tissues through an inhibition of insulin activity, and promote the catabolism of lipids and proteins to be used as gluconeogenic substrates (Sapolsky et al. 2000). We therefore expected to see evidence of energy mobilization in the blood during an acute stress response, which we did with FFAs (Fig. 1b), but not consistently with blood glucose (Fig. 1c).

FFAs increased significantly between baseline and stress-induced levels in both species as expected, given that GCs and adrenocorticotropic hormone (ACTH) promote lipolytic actions (Boonstra and Tinnikov 1998; Sapolsky et al. 2000). While our sample sizes for this variable were small and thus the results should be interpreted with caution, each of the 4 southern flying squirrels and the 3 northern flying squirrels showed increased FFAs over the 30-min period. Our findings are consistent with the 2 previous studies that measured FFAs in response to capture/restraint stress in mammals (Richardson's ground squirrels [*Urocitellus richardsonii*]—Delehanty and Boonstra 2009; platypus [*Ornithorhynchus anatinus*]—Handasyde et al. 2003). We also found species-specific levels of FFAs, with northern flying squirrels having higher baseline levels than southern flying squirrels. Again, this may have to do with differences in tissue sensitivity or metabolism, or perhaps the amount of fatty tissue that each species has available to be mobilized (Delehanty and Boonstra 2011). To our knowledge, it is not yet known if the 2 species store and use fat differently in the fall (the time of year our samples were collected) and winter, but given the differences in latitude and forest type of their respective habitats (Dolan and Carter 1977; Wells-Gosling and Heaney 1984), and the different types and availability of food (Weigl 1978; Vernes et al. 2004), it seems plausible. There may also be differences in absolute amounts of available fat due to body size differences, with southern flying squirrels being smaller and thus having fewer energy stores available. Further molecular and physiological studies would be required to explain these differences in FFA mobilization however.

We found no overall change in blood glucose with acute stress. This was in contrast to previous studies of mammals (*P. hypomelanus* after 60 min of confinement—Widmaier and Kunz 1993; vicunas [*Vicugna vicugna*] after 60 min post-ACTH injection—Bonacic et al. 2003; *U. richardsonii*—Delehanty and Boonstra 2009). Why flying squirrels differed from others species in their response is not clear, although there was interindividual variation in the directionality of response (14 squirrels showed an increase in glucose levels following acute stress, while 7 individuals showed a decrease). This variation may have been due to uncontrolled environmental influences (e.g., timing and type of food ingested or timing of capture during their 3- to 5-h active foraging period), which may have influenced aspects of metabolism, such as insulin activity.

Hematocrit.—Hematocrit may be used as a measure of health condition (Boonstra et al. 2001; Clinchy et al. 2004; Kim et al. 2005) where a low hematocrit may indicate anemia or blood loss because of gastric ulcers. Hematocrit can also be affected in the short term by changes in plasma volume unrelated to anemia (e.g., Dawson and Bortolotti 1997) or by the release of red blood cells from the spleen (Guntheroth and Mullins 1963; Opdyke 1970). Because of these many scenarios, changes in hematocrit should be interpreted with caution.

We found a significant decrease in hematocrit after 30 min of acute stress for both flying squirrel species (Fig. 1d). This contrasts with previous studies in mammals that have found an increase of about 10% (snowshoe hares [*Lepus americanus*]—Boonstra et al. 2001; *V. vicugna* [after 30 min postinjection with ACTH]—Bonacic et al. 2003; meadow voles [*Microtus pennsylvanicus*]—Fletcher and Boonstra 2006) or no effect (*U. richardsonii*—Delehanty and Boonstra 2009). A decrease in hematocrit in flying squirrels following 30 min of acute stress is unlikely to be indicative of anemia or a decline in health. However, the decrease may have been due to a change in plasma volume resulting from blood sampling. For example, hemodilution has been shown to occur in a number of birds and in rats following the drawing of serial blood samples over a short period of time, resulting in rapid decreases in hematocrit (Djojosugito et al. 1968; Kovách and Bálint 1969; Lynn et al. 2003; Dufty 2008). If this were the case in flying squirrels, it suggests they may be more sensitive to hemodilution than most other mammals.

Leukocytes and the N:L ratio.—With measurement of a stress leukogram, the expected response to a stressor is an increase in neutrophils (in mammals) with a concomitant decrease in lymphocytes (Cattet et al. 2003). This is usually reported as an increased N:L ratio (e.g., Kim et al. 2005). During a stress response, there is decreased adherence of neutrophils to vascular walls and thus an increase in circulation (Cronstein et al. 1992), whereas the decrease in lymphocytes is caused by the redistribution of these cells out of circulation and into tissues

(Dhabhar and McEwen 1997). The N:L ratio can therefore change rapidly.

After 30 min of capture stress, there were no clear patterns of change between baseline and stress-induced proportions for any leukocyte type in either flying squirrel species and no differences in proportions of cell types between species (Table 2). This contrasts with previous studies in wild and domestic species, which have reported either an increase (ACTH-treated *V. vicugna*—Bonacic et al. 2003; grizzly bears [*Ursus arctos*]—Cattet et al. 2003; reviewed by Davis et al. 2008), or, in 1 case, a decrease in the N:L ratio following acute stress (e.g., *U. richardsonii*—Delehanty and Boonstra 2009). It is possible that leukocyte responses to stress in some species may occur over longer periods of time than we used (Guthrie et al. 1967; Davis 2005; Mueller et al. 2006), and multiple measurements over several hours might be required to best assess how this part of the immune system is responding to the chosen stressor in flying squirrels.

All individual flying squirrels had N:L ratios well below 1.0, meaning they have many more lymphocytes than neutrophils. These 2 cell types make up the majority of the white blood cell complement in vertebrates (~ 80% combined), but the most abundant leukocyte type varies among taxonomic groups. In most fish, amphibians, reptiles, and birds, the lymphocyte is the most common, whereas in mammals, the neutrophil is most common, but there are exceptions within each group (reviewed by Davis et al. 2008). It appears that flying squirrels are one of these exceptions, along with only a small number of other mammals (mostly rodents) having N:L ratios well below 1.0 (eastern gray squirrels [*Sciurus carolinensis*]—Guthrie et al. 1967; yellow-pine chipmunks [*Tamias amoenus*]—Barker and Boonstra 2005; *M. pennsylvanicus*—Boonstra et al. 2005; cynomolgus monkeys [*Macaca fascicularis*]—Kim et al. 2005; tuco-tucos [*Ctenomys talarum*]—Vera et al. 2013). While it is possible that such white blood cell proportions might be caused by neutropenia (abnormally low neutrophil counts) or lymphocytosis (abnormally high lymphocyte counts), both of which can have a variety of causes, it is not likely that both would occur at the same time, nor that this would be prominent in all 21 individuals sampled. The pattern observed in the New World flying squirrels and other mammalian species and vertebrate groups listed above is most likely the result of evolutionary optimization of the immune system and other related physiology. Further investigation into how these cell types are distributed among tissues and how that distribution changes with stress in these species would be of great value in shedding light on how and why a low N:L (or H:L) ratio is advantageous.

In response to acute stress, 3 of the 5 response variables we measured changed significantly over time in both species of flying squirrels (total cortisol, FFAs, and hematocrit), although not necessarily in directions predicted a priori. When coupled with the exceptionally high baseline cortisol levels, these data suggest that New World flying squirrels may provide an interesting comparative system for the study of stress axis function and its evolution among wild, domestic, and laboratory vertebrates.

Our findings raise some important issues in need of research. We need a better integration of stress physiology with flying squirrel natural history, ecology, and behavior. This can be achieved by looking at how the functionality of the stress axis varies over the biological year so that we can understand how this seasonal variation mediates or is affected by processes like reproduction, juvenile dispersal, and preparing for and surviving winter. For example, could the sensitivity of tissue receptors to cortisol be downregulated differently in northern and southern flying squirrels after breeding to allow for prewinter fattening and differences in FFA utilization and mobilization? As well, knowledge of the basic stress physiology of other flying squirrel genera would provide information about whether the attributes found in New World flying squirrels are genus specific or are characteristic of the monophyletic flying squirrel lineage as a whole. This would shed light on when and why their high baseline cortisol levels may have arisen. These topics will help us to understand how flying squirrels and mammals in general cope with stress in their environment and how the stress axis may have played a role in their evolution.

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