Stress, Metabolism, and Antioxidants in Two Wild Passerine Bird Species

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ABSTRACT

Antioxidants protect against free-radical damage, and free radicals, in turn, are thought to underlie aging. Thus, measuring antioxidants may aid field ecologists in understanding the physiological mechanisms that underlie life-history trade-offs. Antioxidant levels are known to vary markedly in response to the stress of capture in many birds. These changes in antioxidants could result from regulation (e.g., by stress-related hormones) or consumption (e.g., by an increase in free radicals due to increased metabolic rate). Here we experimentally test the effect of increased metabolic rate on circulating antioxidant and corticosterone concentrations in two wild passerine bird species, house sparrows (*Passer domesticus*) and gray catbirds (*Dumetella carolinensis*). We increased metabolic rate via exposure to low ambient temperatures overnight in captivity and measured circulating antioxidant capacity, uric acid, corticosterone, and oxygen consumption in cold-exposed and control individuals. Both species showed increases rather than decreases in all antioxidant parameters overnight, contradicting a consumption-by-energy-expenditure hypothesis. Both positive and negative correlations between antioxidant response and corticosterone response were occasionally but not consistently present, refuting a generalized regulation-by-corticosterone hypothesis. High baseline uric acid predicted diminished response of corticosterone and all antioxidants. Thus, high uric acid may reflect recent stress, poor condition, or a compensatory response. Relationships among metabolic rate, antioxidants, and corticosterone differed qualitatively between the species.

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Introduction

Free-radical damage is thought to underlie much of the aging process (Ames et al. 1993). Aging rates and mortality from extrinsic factors (e.g., climate, predators) vary widely across species and are thought to be key components in the life-history trade-off between investments into current and future reproduction (Ricklefs 1998; Barja 2004). Life-history traits are likely regulated by physiological processes related to the endocrine system, immune function, metabolic rate (Ricklefs and Wikelski 2002), and free-radical balance. Despite their importance in advancing our understanding of the physiological mechanisms underlying life-history strategies, free-radical balance and antioxidant protection are just starting to be explored in an ecological and life-history context (Alonso-Alvarez et al. 2004; Blount 2004; Costantini and Dell'Omo 2006; Hõrak et al. 2006; Rubolini et al. 2006; Tummeleht et al. 2006; Cohen et al. 2007; Isaksson et al. 2007). Here we start testing the connections between energy metabolism, stress, and antioxidants in wild bird species.

Free-radical balance is determined by a number of factors that include rate of radical production, antioxidant defense systems, and effectiveness of damage repair (Halliwell 1994). Although some free radicals can come from exogenous sources, the most abundant free radicals are produced in mitochondria as by-products of respiration in the electron transport chain (Huang and Manton 2004). Free-radical production is thus tied directly (but not necessarily linearly) to metabolism, such that a higher metabolic rate should be accompanied by higher antioxidant demand. However, as the proton gradient across the mitochondrial inner membrane gets stronger, both free-radical production and ATP production efficiency increase, such that free-radical production per unit ATP produced can be downregulated by relaxing the proton gradient across the inner mitochondrial membrane (Brand 2000; Brand et al. 2003; Miwa and Brand 2003). This implies a trade-off between free-radical production and ATP production efficiency, so metabolic rate cannot be taken as a simple proxy for free-radical production. Nevertheless, an acute increase in metabolic rate should result in somewhat increased free-radical production, as implied by upregulation of antioxidants in humans in response to exercise (Ji 1999).

Within antioxidant defense systems, enzymatic antioxidants such as superoxide dismutase and catalase work primarily within mitochondria at the site of free-radical production (Barja 2004), while micromolecular antioxidants such as vitamins E

and C function both in tissues and in the bloodstream (Neuzil and Stocker 1994; Kamzalov and Sohal 2004). In addition, circulating proteins such as albumin have some antioxidant capacity, although it is not clear to what extent this is incidental to other functions, such as hormone transport. Enzymatic antioxidants are highly efficient and specialized, whereas micromolecular antioxidants are more general in their function and can reach small areas the bulkier enzymes cannot (Surai 2002; Huang and Manton 2004). However, while enzymatic antioxidants are basically unchanged when they quench free radicals, micromolecular antioxidants are oxidized in the process and must be regenerated by co-antioxidants or lost. Dietary micromolecular antioxidants include vitamin E and carotenoids, and endogenously produced micromolecular antioxidants include uric acid (UA), glutathione, and, in some species, vitamin C (Surai 2002). UA is by far the most abundant circulating micromolecular antioxidant, and in birds it is also the end product of amino acid metabolism and the main form of nitrogen excretion (Klasing and Austic 1984; Miller et al. 1993).

Previous work has shown that circulating antioxidant levels in birds can change markedly in response to stressors such as capture and handling (Cohen et al. 2007). In a comparison of antioxidant capacity and UA in more than 200 individuals from 57 species sampled immediately at capture and again after 1 h of gentle handling and restraint, the majority of individuals (59%) showed a drop in antioxidant capacity of more than 15%. However, a considerable proportion of individuals (22%) showed the reverse pattern of increasing antioxidant concentrations by more than 15%. Vitamin E and carotenoid levels also decreased after capture stress, but this response was uncorrelated with that of total antioxidant capacity and UA (Cohen 2007; A. A. Cohen and K. J. McGraw, unpublished manuscript). Costantini et al. (2007) reported no effect of capture stress on antioxidant and oxidative stress markers in two migratory passerines, but their data appear to show large, heterogeneous individual responses despite no mean change in each species. However, because this experiment was conducted during migration, a return to resting levels after a long flight could have explained some of the change. A response of antioxidants to stressors is consistent with findings that oxidative stress and enzymatic antioxidants respond to immobilization stress in rats (Liu et al. 1994; Oishi et al. 1999; Gümüslü et al. 2002).

Why do individuals and species differ in their antioxidant response to such stressors? One hypothesis for explaining the decrease in antioxidant concentrations after capture in the majority of species in the previous study is that stress can increase metabolic rate (Sapolsky et al. 2000) and that circulating antioxidants are used up to protect against the high levels of free radicals produced. Hence, differing metabolic responses to stressors could explain the variation in antioxidant responses. Alternatively, antioxidants may be shunted out of the bloodstream to muscle, heart, and lung as part of a concerted suite of changes in response to a stressor. One of the prime mechanisms by which physiological responses to various stressors are regulated in vertebrates is glucocorticoid hormones (Sapolsky et al. 2000). Corticosterone (CORT) is the primary glucocorticoid hormone in birds, and species with different CORT response patterns might thus exhibit different patterns in antioxidant redistribution. In chickens (*Gallus gallus domesticus*), plasma antioxidant capacity and UA increased after CORT administration on timescales of both hours and days (Lin et al. 2004*a*, 2004*b*). The direction of the response in chickens (an increase in antioxidants) is the opposite of that found in most wild species in our previous study. It is not clear whether species differences in antioxidant response to stressors arise from varying regulation strategies or pathways or from differences in stress metabolism and antioxidant consumption.

To start addressing the questions of how antioxidants are regulated and why levels and regulation differ between species, we used overnight exposure to low ambient temperatures to increase metabolic rate in individuals from two passerine species. If antioxidants were consumed in large quantities, we predicted that an increase in metabolic rate would be accompanied by a decrease in antioxidant levels. If antioxidant changes were best explained by regulation independent of metabolic rate, we predicted that changes in antioxidant levels (either up or down) would be consistently correlated with changes in CORT and that this correlation would be stronger than any correlation between metabolic rate and antioxidant change. Long-term exposure to cold stress has previously been shown to result in upregulation of antioxidant enzymes in a small mammal (Selman et al. 2000). Because response of antioxidant systems could differ depending on type of stressor, our results may not be generalizable beyond overnight captivity and cold stress.

We targeted two common wild bird species: house sparrows (*Passer domesticus*) and gray catbirds (*Dumetella carolinensis*). We are aware that two-species comparisons will not allow us to infer adaptive differences (Garland and Adolph 1994), but here we want only to highlight the diversity of antioxidant responses irrespective of selective advantages. House sparrows were thought to have a high tolerance to metabolic stress because they have to withstand cold winter temperatures in their northern temperate habitats, whereas catbirds migrate to the tropics. Stress of captivity was assessed relative to measurements at time of capture, and cold exposure was used to assess the effect of metabolic increase. We were able to definitively reject the consumption hypothesis for these species and to show that CORT regulation also does not adequately explain antioxidant response in either species.

Material and Methods

Birds Captured

Birds were captured with mist nets in and around Princeton, New Jersey, between September 6 and September 15, 2005. Sample sizes for each species are presented in Table 1, not including two cold-exposed house sparrows that were excluded from analyses. All birds were captured before 3 p.m., and most were captured between 6 a.m. and 10 a.m. After capture, birds

Table 1: Low ambient temperature during the night increases oxygen consumption

	Sample Size		Oxygen Consumption ($mL\ O_2$/min, Mean ± SE)			
	Control	Cold	Control	Cold	*T* Statistic	*P*
Gray catbirds	5	6	83.3 ± 2.4	147.7 ± 8.7	−7.11	.0005
House sparrows	11	9	75.8 ± 4.0	131.8 ± 16.6	−6.12	.0001

were housed indoors in a room illuminated by fluorescent lighting in wire cages with water and food (mixed grain and mealworms) ad lib.

Cold Exposure Procedure

Around 10:30 p.m., birds were moved from wire cages to small Plexiglas metabolic chambers to measure oxygen consumption, and lights were turned off. Control birds were kept at room temperature (~25°C). Cold-exposed birds were moved to a neighboring refrigerated room at an ambient temperature of 2°–5°C. Both rooms were maintained dark, and birds were left undisturbed for 12 h.

Metabolic rate during this period was determined by measuring O_2 consumption and CO_2 production in an open-flow, push-through respirometry system (Martin et al. 2003). CO_2 was measured with a CO_2 analyzer (CA-1B; Sable Systems, Henderson, NV), and O_2 was measured with an O_2 analyzer (FC-1B; Sable Systems). For each bird, measurements were taken for 10-min intervals at least six times during the night. Rather than a basal or resting metabolic rate, we took an average metabolic rate (average of all 10-min intervals) because our hypotheses were about antioxidant consumption in relation to total metabolic activity, not the lowest possible "basal" metabolic rate. The average coefficient of variation in metabolic rate across 10-min intervals within individuals was 15%. Birds were released the next morning. All research was conducted with the approval of the Princeton University Institutional Animal Care and Use Committee (IACUC 1516).

Blood Sampling and Measurement of Blood Parameters

Blood samples were taken at three time points: within 3 min of capture, immediately before exposure to cold nighttime temperature in the respiration chambers, and again in the morning immediately after removal from chambers. Blood sampling was done by wing venipuncture. All samples were centrifuged immediately, and the serum was frozen at −80°C until analysis. Circulating antioxidants were assessed with a spectrophotometer using the TEAC procedure as described in Cohen et al. (2007), a technique that measures antioxidant capacity of a liquid to quench free radicals. Measurements have been standardized to Trolox, a water-soluble vitamin E analog and are thus expressed as equivalents of Trolox in millimoles per liter. The assay detects composite antioxidant function of micromolecular antioxidants such as UA, vitamins E and C, and carotenoids but cannot detect antioxidant function of tissue enzymes or other proteins. Thus, although this assay is commonly referred to as "total antioxidant status" (TAS) or "total antioxidant capacity" (TAC), it is not total but reflects only circulating micromolecular antioxidants. To be consistent with previous studies using this assay, we refer to it here as "TAC." UA (mg/dL) was assessed spectrophotometrically using a kit from Sterling Diagnostics (Sterling Heights, MI).

Corticosterone Measurement

We determined plasma concentrations of CORT by direct radioimmunoassay (RIA; modified from Wingfield and Farner 1975 and Tarlow et al. 2001). All samples were analyzed in a single assay. For estimation of recovery, 2,000 dpm of tritiated label was used, and samples were left to equilibrate overnight at 4°C. The next day, samples were extracted once with 4 mL of redistilled dichloromethane. Samples were then dried in a 40°C water bath under a nitrogen stream and redissolved in 550 μL of PBSG buffer. Samples were left to equilibrate with buffer overnight at 4°C. Duplicate fractions of 200 μL were used in the RIA, while fractions of 100 μL were directly counted for the determination of percent recovery after extraction (mean = 89%; CORT antibody B3-163, Endocrine Sciences, Calabasas, CA). Blanks were below detection limit, intra-assay variation for CORT (for a total of nine standards) was 23%, and the lower detection limit was 0.83–1.78 ng/mL.

Data Analysis

TAC and UA were log transformed to achieve normal distributions. Because TAC and level of UA are highly correlated, residual non-UA antioxidant capacity was calculated following Cohen et al. (2007) and indicated abundance of other antioxidants in the system. Changes in antioxidants, UA, CORT, and residual antioxidants were analyzed separately using a repeated-measures design with SAS proc GLM "repeated" statement, with treatment group as a factor.

We predicted that oxygen consumption would be higher in the cold-exposed birds. To distinguish between the hypotheses of consumption and regulation, we statistically examined possible links in the proposed causal chain: increased metabolic rate → increased CORT response → increased antioxidant response. A decrease in antioxidants proportional to metabolic rate would support the hypothesis of direct metabolic consumption, especially if this correlation were stronger than the

metabolic rate-CORT or CORT-antioxidant correlations. A consistent increase or decrease in antioxidants proportional to change in CORT would support a hypothesis of regulation by CORT, and if the change in CORT correlated with metabolic rate, it would confirm the cold exposure as the stressor. If both consumption and regulation are important, antioxidants should decrease and all variables should correlate.

We used ANCOVAs (proc GLM, SAS ver. 9.1; SAS, Cary, NC) to examine effects of CORT and metabolic rate on antioxidant response. The final postchamber values of antioxidant variables were used as dependent variables, and the model included the baseline value, the metabolic rate (oxygen consumption), and the CORT response (measured as postchamber minus baseline). The associations between metabolic rate and CORT response were assessed with Pearson's correlation coefficients (SAS proc corr).

Finally, we assessed whether baseline values of physiological variables might indicate overall health status of the birds and thus predict the magnitude of the overall response. This was done by examining correlation tables for any pretreatment parameters that showed a large number of significant correlations with posttreatment parameters. Any such pretreatment parameters were then analyzed further using ANCOVAs to control for treatment effects. To minimize Type I error, we tested only ANCOVAs in baseline parameters that showed significant correlations with multiple independent response variables.

Results

Baseline Values of CORT and Antioxidants

In both species, TAC and UA at time of capture were highly correlated ($r = 0.76$, $P < 0.0001$ in house sparrows; $r = 0.87$, $P = 0.0005$ in gray catbirds). Furthermore, UA was negatively correlated with the residual antioxidant capacity (RES; $r = -0.54$, $P = 0.008$ in house sparrows; $r = -0.71$, $P = 0.01$ in gray catbirds). In combination, these results suggest that under baseline (nonstressed) conditions, antioxidant capacity is largely accounted for by UA in both species. Baseline CORT was positively associated with baseline UA in house sparrows ($r = 0.45$, $P = 0.05$) but not in gray catbirds ($r = -0.18$, $P = 0.59$). No other baseline measures were correlated in either species.

Low Ambient Temperatures Increase Metabolic Rate

In both species, birds exposed to low ambient temperatures had oxygen consumption nearly double that of individuals kept at room temperature (Table 1). Two birds did not follow this pattern: the two lowest metabolic rates recorded were in cold-exposed house sparrows. We are confident that the measurements were accurate and assume that these birds went hypothermic. For consistency, the two birds were excluded from all analyses.

Antioxidant and CORT Response to Low Ambient Temperatures and Captivity

During the course of the experiment, all antioxidant parameters (TAC, UA, and RES), as well as CORT, showed time-of-measurement effects in both species, almost always an increase (repeated-measures ANCOVAs; Table 2). UA values increased about fourfold. For both TAC and UA, there was a significant effect of time × treatment group in house sparrows, indicating that the increase in TAC and UA was confined to cold-exposed house sparrows (Fig. 1*a*, 1*c*). In contrast, there was no time × treatment interaction in gray catbirds, indicating that temperature treatment had no effect on TAC or UA in this species (Fig. 1*b*, 1*d*). CORT concentrations increased in both species and treatment groups over time, with a nonsignificant trend ($P = 0.08$) toward a greater increase in cold-exposed

Table 2: Mean values (±SE; untransformed) and effect sizes of changes in blood parameters

	Capture	Evening	Postchamber	Time Effect (F)	Time × Treatment Effect (F)
Gray catbird:					
TAC	.93 ± .12	1.41 ± .09	2.40 ± .30	7.40*	1.05
UA	8.73 ± 2.89	24.3 ± 2.02	40.2 ± 4.43	10.45**	.71
RES	.06 ± .04	−.05 ± .01	.05 ± .03	7.73*	.16
CORT	1.30 ± .28	37.7 ± 5.5	71.4 ± 7.70	37.0***	3.39
House sparrow:					
TAC	.77 ± .03	1.45 ± .07	1.94 ± .26	61.9***	7.98**
UA	6.48 ± .07	21.1 ± 1.43	30.2 ± 4.56	57.2***	7.59**
RES	−.03 ± .01	−.01 ± .01	.02 ± .02	3.67*	4.19*
CORT	1.10 ± .25	26.7 ± 2.6	47.7 ± 6.5	47.6***	2.83

Note. From repeated-measures ANCOVA. CORT = corticosterone; RES = residual antioxidant capacity; TAC = total antioxidant capacity; UA = uric acid.

* $P < 0.05$.

** $P < 0.01$.

*** $P < 0.0001$.

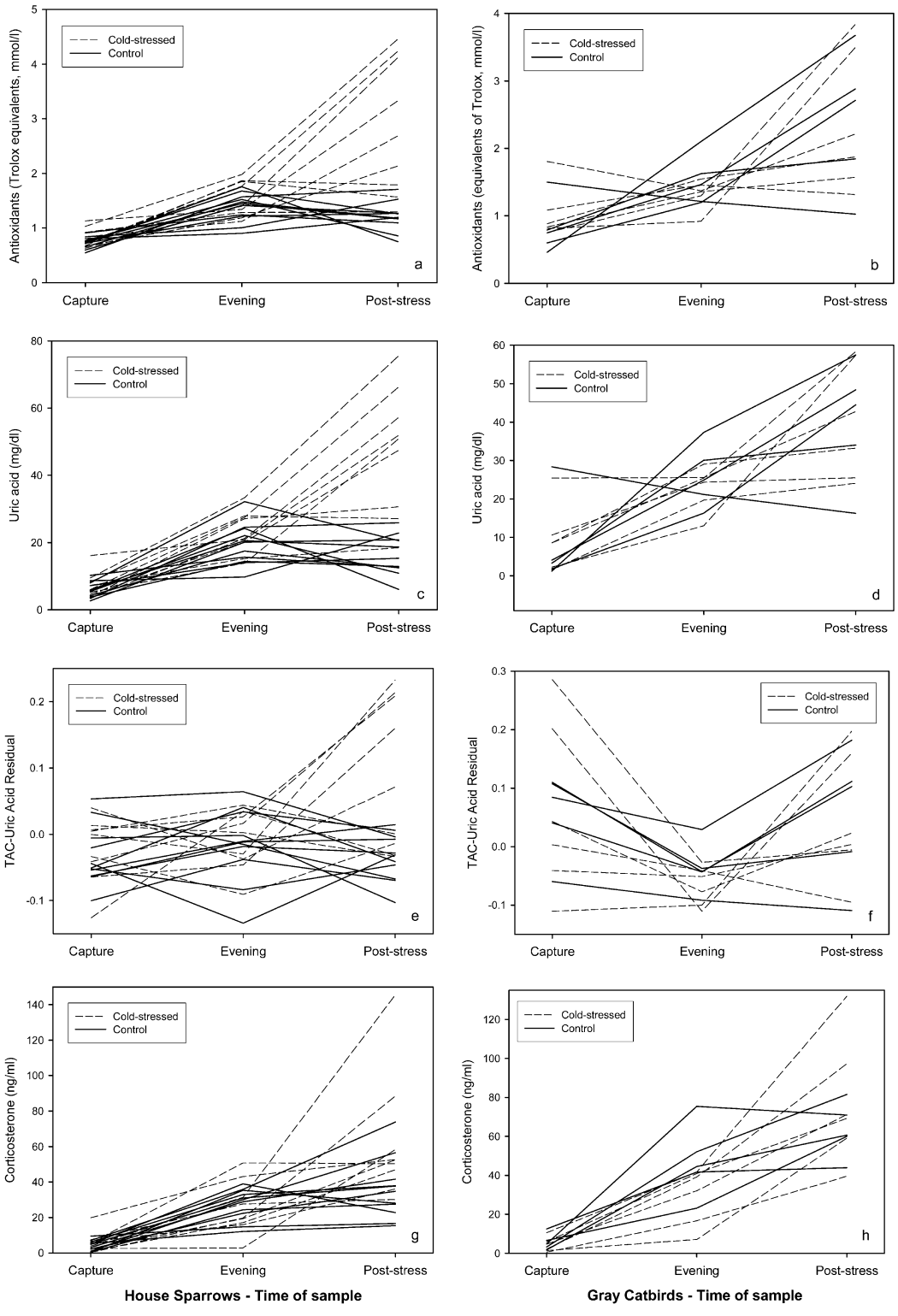

Figure 1. Change in antioxidants and uric acid in individual birds over the course of treatment. *a*, Total antioxidant capacity (TAC) in house sparrows; *b*, TAC in gray catbirds; *c*, uric acid (UA) in house sparrows; *d*, UA in gray catbirds; *e*, TAC-UA residual in house sparrows; *f*, TAC-UA residual in gray catbirds; *g*, corticosterone in house sparrows; *h*, corticosterone in gray catbirds. A repeated-measures ANOVA shows highly significant effects of sample time on all variables for both species, except for residual antioxidant capacity (RES) in house sparrows. The time × treatment interaction is significant only for TAC, UA, and RES in house sparrows, showing that the large increases in these variables occur in the cold-exposed individuals.

Table 3: Portion of variance in responses of antioxidant measures explained independently by corticosterone (CORT) response and oxygen consumption

	TAC	UA	RES
Gray catbird O_2:			
r^2	.05	.09	.06
P	.45	.38	.47
Gray catbird CORT:			
r^2	.002	.009	.28
P	.88	.77	.14
House sparrow O_2:			
r^2	**.28**	**.29**	**.32**
P	**.0005**	**.0008**	**.004**
House sparrow CORT:			
r^2	**.20**	**.21**	.09
P	**.002**	**.003**	.09

Note. From ANCOVAs, Type III sum of squares (SS). CORT response is postchamber minus baseline; models use postchamber antioxidant value as the dependent variable, with baseline antioxidant level, CORT, and oxygen consumption (O_2) as independent variables. r^2 = partial Type III SS/total SS. RES = residual antioxidant capacity; TAC = total antioxidant capacity; UA = uric acid. Values in boldface are significant at $P = 0.05$.

birds (Fig. 1*g*, 1*h*). RES followed a different pattern (Table 2). In gray catbirds, there was no net increase over time (Fig. 1*f*). In contrast, house sparrows showed a significant overall increase in RES, with an even stronger increase in the cold-exposed birds (Fig. 1*e*). In summary, for all three antioxidant measures, house sparrows showed large overnight increases only in cold-exposed birds, whereas gray catbirds showed no evidence of a treatment effect (Table 2). We would like to highlight the considerable heterogeneity of responses of individuals within treatment groups (Fig. 1).

Testing the Antioxidant Consumption Hypothesis

The consumption hypothesis predicts that increased metabolism results in decreased antioxidant levels. Our data were inconsistent with this hypothesis: antioxidants increased in both species under conditions of increased energy expenditure. Both oxygen consumption and CORT response were positively associated with response of TAC, UA, and RES in house sparrows but not in gray catbirds (Table 3). In other words, house sparrows with a high metabolic rate (i.e., cold-exposed birds) showed large increases in all antioxidant parameters overnight.

Testing the Antioxidant Regulation Hypothesis

The regulation hypothesis predicts that increased CORT levels induced by stress result in a correlated change in antioxidant levels independent of the energy expenditure of an organism. CORT change was not consistently correlated with antioxidant change in our data. In gray catbirds, the CORT response to captivity (i.e., difference between capture and evening measurements) correlated positively with the response to captivity of TAC ($r = 0.64$, $P = 0.03$) and UA ($r = 0.62$, $P = 0.04$) but not RES ($r = -0.03$, $P = 0.93$). In house sparrows, it correlated negatively with RES ($r = -0.57$, $P = 0.008$) but not TAC ($r = -0.23$, $P = 0.33$) or UA ($r = 0.04$, $P = 0.87$).

Postchamber levels of all blood parameters were much higher than evening levels (Table 2), implying additional stress from either cold exposure (house sparrows) or some unknown factor (gray catbirds). Controlling for metabolic rate, house sparrows with a large overnight CORT response showed concomitant increases in all antioxidant parameters, but there was no relationship between CORT response and overnight change in antioxidant parameters in gray catbirds (Table 3). However, in both species, we found a nonsignificant trend between overnight CORT response and overnight change in RES. We detected no correlation between metabolic rate and CORT response in either species (for gray catbirds, $r = 0.26$, $P = 0.44$; for house sparrows, $r = 0.26$, $P = 0.27$). Taken together, these results do not support the regulation hypothesis: often, antioxidant change is uncorrelated with CORT change, and when it is, the direction is not consistent. In addition, the CORT-independent correlation between antioxidant change and metabolic rate in house sparrows does not support CORT as the primary regulator of antioxidants.

Do Baseline Antioxidant Levels Predict Antioxidant and CORT Responses?

We tested whether pretreatment (baseline) antioxidant levels correlated with the magnitude of the overnight response. Only baseline UA consistently predicted the overnight response in antioxidants and CORT (we present response as postchamber minus baseline value and control for temperature treatment; Table 4). The predictive power of baseline UA was strong in gray catbirds but marginal in house sparrows (Fig. 2).

Discussion

We found that house sparrows and gray catbirds increased levels of CORT as well as antioxidants during overnight captivity.

Table 4: Baseline uric acid (UA) predicts overnight response of corticosterone (CORT) and antioxidants

	TAC	UA	RES	CORT
Gray catbirds:				
r^2	**.52**	**.89**	.03	**.55**
P	**.01**	**<.0001**	.61	**.007**
House sparrows:				
r^2	.08	**.35**	.08	.13
P	.1	**.0004**	.15	.09

Note. Columns show association of baseline UA with overnight response of variables in column headings. All data are from ANCOVAs controlling for treatment. r^2 = partial Type III sum of squares (SS)/total SS. TAC = total antioxidant capacity; RES = residual antioxidant capacity. Values in boldface are significant at $P = 0.05$.

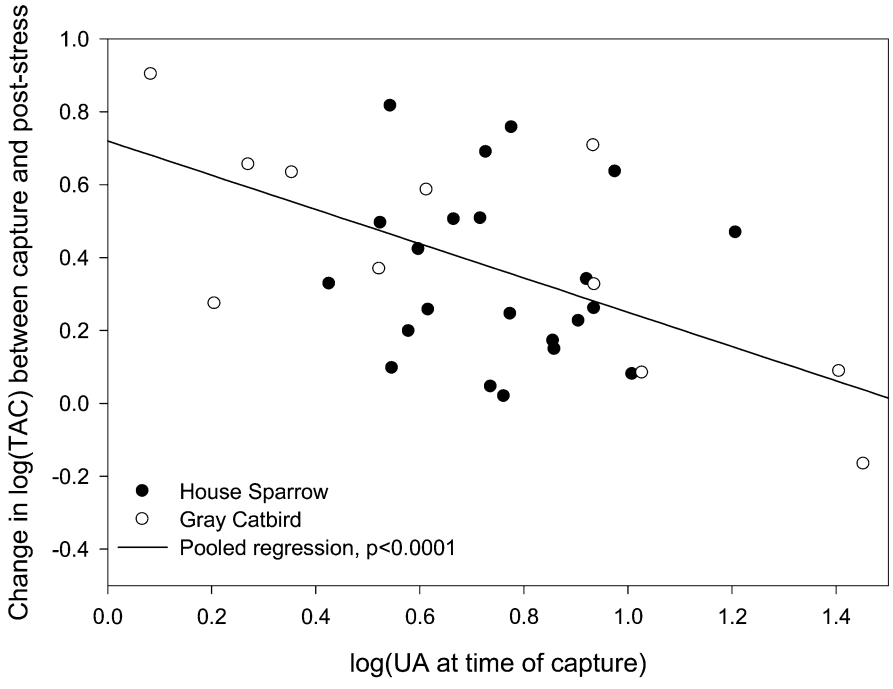

Figure 2. Uric acid (UA) at capture predicts response of total antioxidant capacity (TAC) to overnight captivity. Higher baseline uric acid is associated with lower response to captivity stress. Similar patterns are seen for response of corticosterone and uric acid. House sparrows follow the general pattern, but the distribution of initial values is narrower, making the response in this species alone nonsignificant.

When individuals were additionally challenged metabolically, they increased their antioxidant levels even more in one of the two species investigated here. These results are in apparent disagreement with previous observations showing a general decline in antioxidant capacity after 1 h of captivity, and the largest increases here were several-fold higher than in that larger sample (Cohen et al. 2007). The increase in antioxidant measures after a night of captivity suggests that metabolic consumption is not the main cause of micromolecular antioxidant fluctuations in response to overnight captivity or cold exposure. We did not find consistent correlations between antioxidants and CORT, so it is unlikely that CORT directly or primarily regulates antioxidant flux. We therefore reject both of our mechanistic hypotheses for antioxidant response to external stressors: (i) the "consumption hypothesis" that predicted a decrease of antioxidants when metabolism is increased and (ii) the "corticosterone regulation hypothesis" that predicted a simple regulation of antioxidants by CORT.

Increases versus Decreases in Antioxidant Levels

We see three possible explanations for the discrepancy in the direction of antioxidant change between this study and the majority of species studied by Cohen et al. (2007). First, the two species in this study might, by chance, be unrepresentative of larger trends across species. Second, birds might respond differently to being held in a cloth bag versus in a cage. That is, individuals may perceive these different treatments as differentially stressful. Consistent with this, in this experiment, gray catbirds showed no difference in response between cold-exposed and control groups but did show a difference between response to captivity (i.e., by evening measurement) and response to being put in the metabolic chamber. Third, the time course of antioxidant response to stressors may always involve an initial drop (within an hour), followed by an increase to above baseline levels as UA is regenerated. Variation in this time course would explain the heterogeneity of responses observed here and by Cohen et al. (2007). In any case, the increase in antioxidants observed here contradicts the hypothesis that increased metabolic rate drives a net loss of antioxidants as they quench free radicals.

Passive versus Active Increases in Antioxidants

Antioxidants could increase because they are upregulated (active increase). For example, increases in antioxidant enzymes as part of cold acclimation are presumably a result of regulation (Selman et al. 2000). Alternatively, increases in antioxidants could be produced as a by-product of enhanced metabolism (passive increase). For example, increased UA in fasting ducks has been attributed to protein catabolism (Hollmén et al. 2001). We cannot exclude a passive increase for UA as a result of increased protein catabolism. However, all antioxidants increased simultaneously, even those that are not by-products of catabolic processes. In particular, TAC-UA residuals, representing the sum of all non-UA circulating antioxidants, in-

creased in parallel with UA. TAC-UA residuals theoretically should relate to levels of antioxidants such as vitamin E and carotenoids but actually are associated more consistently with TAC and UA, with considerable variation across species (Cohen 2007). We have shown that vitamin E and carotenoids more consistently decrease after 1 h of captivity than TAC and UA, consistent with the inability of birds to synthesize these antioxidants de novo (Cohen 2007; A. A. Cohen and K. J. McGraw, unpublished manuscript).

Regulation of Antioxidants in Response to Stressors

Because increases in antioxidant levels are not solely attributable to passive increases, regulatory systems are almost certainly involved. Relative metabolic usage of various tissues should change in response to stressors, so there is good reason to expect shunting of antioxidants between tissues and thus changes in circulating levels. Moreover, there may be a compensatory response such that oxidative stress (or the likelihood of imminent oxidative stress) induces production of endogenous antioxidants. Such a response in antioxidants has been shown after acute and chronic exercise in humans (Ji 1999). CORT seemed the most likely regulatory mechanism, given its known effects on immune and metabolic processes (Wingfield et al. 1998). In chickens, CORT alone was sufficient to trigger large increases in UA and antioxidant capacity (Lin et al. 2004*a*, 2004*b*). However, our results do not support CORT as a primary regulator of antioxidants. Some measures of antioxidant response to captivity were correlated with the CORT response, but many were not, and the direction of the correlation varied. Patterns differed between species, and in both species, the associations differed for captivity stress (i.e., by evening) and for overnight stress in the metabolic chambers.

Such inconsistent results suggest a noncausal association: CORT likely correlates with other regulators of stress response some of the time. However, we cannot exclude two more complex scenarios for regulation of antioxidants by CORT. First, CORT regulation of antioxidants could be context dependent (Gill et al. 2007). Both physiological and environmental factors could be part of such context dependency. For example, antioxidants might be subject to CORT regulation only when levels are sufficiently high or the direction of the regulation might depend on season. Second, lack of complete time series data here might have obscured lag effects in the regulation. In this case, the effect of CORT on antioxidants could be either delayed or long-lasting, such that a change in CORT produces a change in antioxidants only after several hours, or antioxidants remain at elevated levels even after CORT has returned to its baseline level. Because our experimental design was based on pairing antioxidant and CORT levels at a few time points, we were not able to test hypotheses of such complex regulation. Finally, the sympathetic nervous system may play a role in regulating antioxidants, although we know of no data on this subject.

There is considerable heterogeneity not only in antioxidant-CORT associations but also in responses of different antioxidants to stress. Even within the same individual, different antioxidant parameters often respond differently to the same stress (Cohen 2007; A. A. Cohen and K. J. McGraw, unpublished manuscript). Such differences in regulation could be explained by context specificity, by target specificity, or by multiple regulatory mechanisms. Context specificity would involve a given regulatory molecule having effects only in certain species or certain environments, such as seasonal and sex differences in effects of sex hormones (Gill et al. 2007). Target specificity would involve one regulatory molecule having different effects on different antioxidants. In house sparrows, although CORT response was associated with antioxidant response, metabolic rate was associated with antioxidant response independently of CORT, suggesting multiple regulatory mechanisms.

Uric Acid and Its Relation to Stress

It is remarkable that baseline UA (at capture) significantly predicted overnight responses of antioxidants and CORT. High baseline levels of UA were associated with a weaker overnight response in antioxidants and CORT. Potentially, elevated baseline UA levels indicate poor health and little ability to further increase levels because UA must be kept at concentrations low enough to avoid gout. That is, even if UA has beneficial effects as an antioxidant, high levels may indicate a compensatory response to stress, and there may be a ceiling on these levels imposed by the threat of gout (Guo et al. 2005). Furthermore, although UA is a potent antioxidant, its physiological relevance is not well established. In circulating systems, protection of lipids is of primary importance, and thus the lipid-soluble antioxidants may be more important than UA (Neuzil and Stocker 1994; Niki 2004).

In greenfinches (*Carduelis chloris*), high UA levels were associated with high levels of lipid peroxidation (Hõrak et al. 2007), and in Leach's storm petrels (*Oceanodroma leucorhoa*), UA was lower in older and more reproductively successful individuals (A. A. Cohen, unpublished data). In white-crowned sparrows (*Zonotrichia leucophrys*), UA increased after exercise (Tsahar et al. 2006). Across species, UA is lower in longer-lived species with a slower pace of life (Cohen et al., forthcoming). There is thus a growing body of evidence suggesting that elevated UA, even below the levels that cause gout, is an indicator of poor condition (but see Simoyi et al. 2002). The ability to respond to a stimulus and subsequently to restore homeostatic balance is considered a marker of health in studies of aging and chronic stress (Lipsitz 2002), so a less robust response of UA to stressors when its levels are elevated is consistent with high UA indicating poor health.

The two passerine species investigated here differ markedly in their physiological response to stressors, and there is also considerable variation within each species. For example, gray catbirds have a wider range of baseline antioxidant levels than house sparrows, consistent with previous data showing a wide intraspecific range for levels of antioxidants in gray catbirds

(Cohen 2007). Thus, while antioxidants clearly show dramatic regulated responses to stressors, the nature of the response and its regulation appear to depend on multiple factors, including type of stressor, type of antioxidant, species, condition, and individual characteristics. A fuller understanding of the role and regulation of antioxidants during stressful situations will depend on knowledge of these factors and their interactions.

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