Increased Energy Expenditure but Decreased Stress Responsiveness during Molt

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ABSTRACT

Baseline and stress-induced corticosterone (CORT), heart rate (f_H), and energy expenditure were measured in eight captive European starlings *Sturnus vulgaris* during and following a prebasic molt. The f_H and oxygen consumption ($\dot{V}O_2$) were measured simultaneously across a range of heart rates, and energy expenditure (kJ/d) was then calculated from data. Energy expenditure and f_H were strongly and positively correlated in each individual. Baseline f_H and energy expenditure were significantly higher during molt. Molting starlings expended 32% more energy over 24 h than nonmolting birds, with the most significant increase (60%) occurring at night, indicating a substantial energetic cost to molt. Furthermore, the cardiac and metabolic responses to stress during molt were different than during nonmolt. Birds were subjected to four different 30-min acute stressors. The f_H and CORT responses to these stressors were generally lower during molt. Although restraint caused a 64% increase in daily energy expenditure during nonmolt, no other stressor caused a significant increase in energy expenditure. Overall, our data suggest that molt is not only energetically expensive but that it also alters multiple stress response pathways. Furthermore, most acute stressors do not appear to require a significant increase in energy expenditure.

Introduction

Molt, or the routine process of replacing worn feathers, is important for birds because it restores flight and thermoregulatory performance (Swaddle et al. 1999). Most north-temperate passerines undergo a complete prebasic molt (the loss and regrowth of all feathers) following the breeding season, which requires metabolic changes and increased energy expenditure (King 1981; Murphy and King 1992; Lindström et al. 1993; Klaassen 1995). In birds, energy expenditure has been measured with a variety of techniques (reviewed in Murphy 1996). One common technique is to calculate energy expenditure from the rate of oxygen consumption ($\dot{V}O_2$). Direct measures of $\dot{V}O_2$ require placing the animal in a metabolic chamber, which constrains the animal's movement. To avoid the consequent risk of nonrepresentative behavior, many studies have used doubly labeled water (DLW) to measure $\dot{V}O_2$ indirectly. DLW estimates CO_2 production, which later can be used to estimate energy expenditure; with this technique, the animal can move freely (see review of validation and technique in Roberts 1989). Several recent studies have estimated $\dot{V}O_2$ and energy expenditure using the relationship between heart rate f_H and $\dot{V}O_2$ (Nolet et al. 1992; Butler 1993; Bevan et al. 1994, 1995); this technique also allows the animal to move freely. Butler et al. (2004) reviewed the literature regarding the accuracy of the DLW technique and the f_H method and found that both are accurate in that an estimate of the mean metabolic rate of a group of animals is comparable with the metabolic rate measured directly. In addition, both techniques can be used to measure short-term energetic costs of stress. One major advantage to the f_H method over the DLW technique is that the f_H method can be used to accurately assess energetic costs of specific activities over a long period of time (Butler et al. 2004). In this study, we validated the f_H method for use in European starlings *Sturnus vulgaris*. After calibrating $\dot{V}O_2$ and f_H, we used f_H to estimate baseline and acute stress-induced energy expenditure in molting and nonmolting starlings. Although we focused on short-term stressors, the results of this study could be used to compare the energetic costs of long-term stress during molt and nonmolt, which would benefit from the use of the f_H method rather than the DLW technique.

In addition to incurring energetic costs, birds may incur other physiological costs when molting. For example, in many passerine birds, baseline and stress-induced corticosterone levels (CORT, the avian glucocorticoid; Holmes and Phillips 1976) are reduced during molt compared with other times of the year (reviewed in Romero 2002). An attenuation of catabolic hormones such as CORT may be needed for the anabolic process of feather growth. Studies of captive European starlings have shown that CORT is significantly lower during molt than during simulated summer (long-day light cycle) and winter (short-day light cycle; Romero and Remage-Healey 2000), yet CORT and insulin retained their opposing regulatory roles over glu-

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cose (Remage-Healey and Romero 2000). Even though the baseline metabolic actions of CORT remain consistent during molt, CORT's actions during stress may be altered. The attenuated baseline and stress-induced CORT levels during molt may be an adaptation to avoid potential deleterious effects of CORT on feather growth since CORT is known to inhibit protein synthesis and stimulate proteolysis (Sapolsky et al. 2000).

Although it is tempting to conclude from the attenuated CORT release that the entire stress response is downregulated during molt, corticosterone release is only one pathway initiated during stress. CORT release occurs several minutes after the initiation of an acute stressor and thus constitutes only the second wave of the stress response. The first wave of the stress response is regulated by the sympathetic nervous system, directly by sympathetic innervation of the heart and indirectly through the release of the catecholamines epinephrine and norepinephrine from the adrenal gland, which results in a rapid increase in heart rate, among other physiological responses (Sapolsky et al. 2000).

In this study, we used European starlings to test the hypotheses that molt alters both the baseline and the acute stress response and that baseline and stress-induced heart rate and energy expenditure change in a manner corresponding to changes in CORT. We have demonstrated that during acute stress, f_H is under sympathetic control, and thus, f_H provides an accurate estimate of the first wave of the stress response (Cyr et al., forthcoming). Consequently, we used f_H to estimate the sympathetic response to an acute stressor. Our goals were to (1) estimate the baseline cardiac response and energetic cost of molt, (2) investigate whether CORT release is the only aspect of stress modulated during molt or whether other pathways are altered as well, and (3) estimate the energetic cost of various acute stressors and determine whether these costs change during molt.

Material and Methods

Animals

European starlings are a commonly used laboratory species with extensive data available on their response to stress during molt (e.g., Romero and Remage-Healey 2000). Starlings undergo a complete prebasic molt that lasts about 85 d in captivity and 100 d in the wild (Rothery et al. 2001). Also, starlings are large enough (about 80 g) to accept heart monitor implants and retain a wide range of motion and flight (Nephew et al. 2003).

We captured nonbreeding European starlings in eastern Massachusetts during the winter and immediately transported them to an indoor flight aviary at Tufts University (Medford, MA), where they were held on a short-day light cycle (11L : 13D) to mimic winter conditions. The f_H transmitters were implanted into 16 starlings—four males and four females for the calibration experiment and three females and five males for the molt versus nonmolt comparison. These birds were then moved to an experimental room maintained at 25°C and placed in individual cages (45 cm × 37 cm × 33 cm) arranged so they were able to see and hear each other. They were given at least 7 d to recover before the initiation of the calibration experiment.

For the molt versus nonmolt comparison, birds were exposed to a long-day light cycle (19L : 5D) to mimic summer conditions for 6 wk in the aviary. Then the birds were shifted to a short-day light cycle, which stimulated a prebasic molt. The f_H transmitters were implanted at least 7 d after molt initiation, after which the birds were moved to individual cages in the experimental room to recover from surgery. The experimental protocol was implemented approximately 60 d after molt initiation (when birds were replacing their eighth or ninth primaries). Although the molt portion of this study was completed before the nonmolt portion, both the CORT titers and the f_H levels from both conditions during this study were comparable with CORT titers and the f_H levels from molting and nonmolting starlings found in other studies in our laboratory, suggesting that birds that were exposed to various stressors during molt did not alter their responses during nonmolt.

Food (Start and Grow, Purina Mills, St. Louis, MO) and water were provided ad lib. to all birds at all times. All experiments were conducted according to Association for Assessment of Laboratory Animal Care guidelines and approved by the Institutional Animal Care and Use Committee at Tufts University.

Heart Rate and Activity Data Collection

We collected f_H and activity data using Data Sciences International TA 10EA-F20 transmitters (St. Paul, MN). The transmitters sent signals to a receiver positioned on one side of each bird's cage. The data were then transferred to a computer equipped with Dataquest Advanced Research Technology Gold 1.1 software, which records continuous electrocardiogram (ECG) signals and calculates the mean *R* wave frequency over 10-s intervals. For stress-induced f_H during the sample period, the mean f_H in beats per minute (BPM) was calculated across 30 s. Overnight f_H was collected in 300-s averages. The f_H recovery time was defined as the time it took for the birds to return to within 1 standard error of their average baseline f_H following each stressor.

Activity was estimated by the receiver measuring changes in signal intensity from the transmitter. Activity data are base on the signal strength. The signal is sent from the transmitter to two antennas on the receiver (placed on the outside of the cage). This is a continuous signal, and as the bird moves, the angle of the signal to the two antennas changes. The activity data are derived from these changes. Thus, each measurement is a relative value; therefore, there are no units given to activity level.

Heart Rate Transmitter Implantation

We anesthetized starlings by injecting Ketamine (30 mg/kg) and Xylazine (10 mg/kg) into the pectoral muscle. For a detailed description of implantation, see Nephew and Romero (2003).

Briefly, transmitters and all surgical instruments were sterilized using MaxiCide sterilizing and disinfecting solution (Henry Schein, Melville, NY). For the surgery, a 15-mm incision was made in the abdomen and followed by 5-mm incisions made in the neck and adjacent to the pygostyle, where ECG leads surrounded by flexible polyurethane were placed. The ECG leads were slid through the 15-mm incision to the 5-mm incisions using a trochar and sleeve and then sutured twice to the muscle. The ends of the leads were placed under the skin, after which the 5-mm incisions were sutured closed. A 14-mm incision was made in the muscle of the abdominal wall and the body of the 4.0-g transmitter (20 mm × 10 mm × 10 mm) was inserted into the abdominal cavity along with any excess leads. The muscle of the abdominal wall was then sutured closed, followed by the skin. Antibiotic ointment was placed on all incision areas. Experiments began at least 7 d postsurgery to ensure recovery.

Calibration of Heart Rate versus Oxygen Consumption (Respirometry)

Heart rate measurements allow us to indirectly estimate $\dot{V}O_2$ and, ultimately, energy expenditure, because the two variables are related to each other, as derived by Fick's equation: $\dot{V}O_2 = f_H\dot{V}_s(C_{aO_2} - C_{\bar{v}O_2})$, where $\dot{V}_s$ is cardiac stroke volume, C_{aO_2} is the oxygen content of arterial blood, and $C_{\bar{v}O_2}$ is the oxygen content of mixed venous blood (Fick 1870). If the oxygen pulse, $\dot{V}_s(C_{aO2} - C_{\bar{v}O_2})$, remains constant, there is a linear relationship between f_H and $\dot{V}O_2$, and the former can be used to determine the latter (Green et al. 2001). However, calibration experiments to determine the exact relationship between the two variables need to be conducted. Calibrations were performed on eight birds between November 8 and 15, 2005. Birds with implanted f_H transmitters were placed in 2-L plastic metabolic chambers to simultaneously measure f_H and $\dot{V}O_2$ in 10-s intervals. On average, birds were held in the respirometry chamber for a period of 2.3 ± 0.6 h (mean ± SE). In the chamber, birds could freely move around, but when lights were off, they were generally quiescent. During daytime trials, the birds were placed in the chamber and left alone for 1 h, and then the birds were subjected to an acute stressor; specifically, the door to their room was slammed five times over 2 min. The birds were then left alone for the remainder of the daytime trial. Birds were left alone for the entire duration of the nighttime trial. Thus, the daytime and nighttime trials provided an estimate of $\dot{V}O_2$ over a range of heart rates.

We measured oxygen consumption and carbon dioxide production ($\dot{V}CO_2$) in an open-flow, push-through respirometry system. External air was dried in Drierite columns and pumped through a mass flow controller (TR-FCI, Sable Systems, Las Vegas, NV) and a multiplexer (V2-0, Sable Systems) into the metabolic and reference chambers. Flow rate was 1,000 mL/min, and the flow controller was calibrated before use via a bubble meter. A factory calibration after use indicated that flow rate errors were less then 1.2%. Air leaving the chambers was dehumidified using a Peltier-effect condenser (PC-1, Sable Systems), and CO_2 concentration was measured from a subsample of the outlet flow (CA-1B, Sable Systems). Before $\dot{V}O_2$ was determined (FC-1B, Sable Systems), Drierite was used to scrub any remaining water from the air. The respirometry system was tested for leaks by pressurizing it and determining that no air was lost after 10 min of observation.

We estimated instantaneous oxygen consumption, using the equation of Bartholomew et al. (1981):

$$FEO_2(\text{eq}) = FEO_2(t-1) + \left[\frac{FEO_2(t) - FEO_2(t-1)}{1 - e^{(-\dot{V}/V)\Delta t}}\right],$$

where FEO_2 is the oxygen concentration in the excurrent air, V is the volume of the system (including tubing), $\dot{V}$ is the flow rate through the system, and Δt is the interval between measurements at times t and $t-1$. We determined the denominator of Bartholomew et al.'s (1981) equation (the so-called Z value) empirically using Datacan (Sable Systems). We then calculated rate of oxygen consumption with equation (4a) of Withers (1977):

$$\dot{V}O_2 = \dot{V}\left[\frac{FIO_2 - FEO_2(\text{eq})}{1 - FIO_2}\right],$$

where FIO_2 is the incurrent oxygen concentration. We also determined the respiratory quotient—the ratio of CO_2 produced to O_2 consumed—and used it to calculate thermal equivalents and metabolic rate (kJ/d; following Walsberg and Hoffman 2005).

To relate f_H to $\dot{V}O_2$, we temporally aligned both data files and randomly selected seven time points during the ca. 60-min measurement period. Thus, calibration estimates were ca. 10 min apart, which should have ensured that there was complete air turnover in the measurement chamber. Furthermore, there was no temporal autocorrelation of metabolic and f_H values over that duration; thus, we assume that these seven data points are independent for statistical analysis within an individual bird. All energy expenditure data we present for molting and nonmolting birds were converted from f_H to energy expenditure using the regression equation derived from the relationship between energy expenditure and f_H, seen in Figure 1.

Experimental Procedure

The eight molting starlings were split into two groups of four. Basal f_H was measured over 24 h before beginning of the 8-d stressor session for both molt and nonmolt. Body mass (M_B, to the nearest 0.5 g) was measured 1 d before beginning the 8-d stressor session for both molt and nonmolt. On the day a stressor was applied, blood samples were taken from the brachial vein at 1200 hours, within 3 min of entering the experimental room to measure basal CORT levels. Immediately fol-

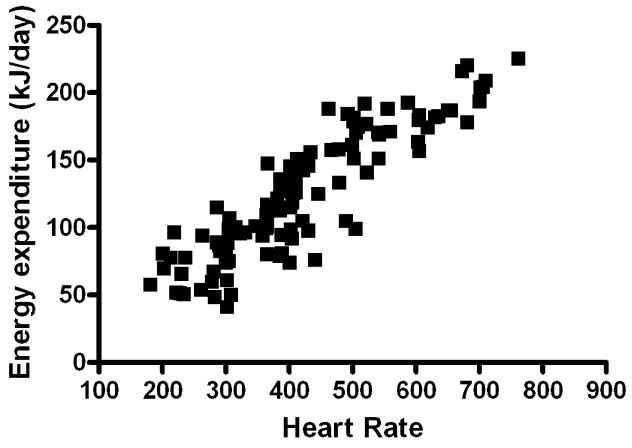

Figure 1. Relationship between heart rate (f_H; beats/min) and energy expenditure (kJ/d) of eight starlings (14 measurements from each bird: seven during the day and seven during the night). The regression equation is $\dot{V}O_2 = 0.306f_H - 3.38$, $r^2 = 0.798$.

lowing the baseline blood sample, the stressor was applied. The starlings were then subjected to one of four 30-min acute stressors every other day: two degrees of acute density stress (adding one or five novel birds to a resident's cage), restraint, or an auditory stressor (loud music).

The restraint stressor consisted of placing each bird in an opaque bag, which was hung from the top of the bird's cage in order for the f_H signal to be received. The auditory stressor was a loud radio tuned to a local radio station and switched on remotely. For a detailed description of the cage design used for the intruder stressors, see Nephew and Romero 2003. In brief, two wire cages (45 cm × 37 cm × 33 cm) were interlocked one on top of the other with a trap door between the cages. Both cages contained a perch, and the f_H receiver and the food and water dishes were located in the upper cage. The resident bird was placed in the upper cage, while one or five birds were placed in the lower cage. One hour later, the trap door was opened remotely using fishing line and then closed after the bird(s) flew into the upper cage. The f_H estimates were taken within 30 s of all birds entering the top cage.

Groups of four birds received a stressor on each day in a staggered fashion (birds 1–4 on day 1, birds 5–8 on day 2, birds 1–4 on day 3, etc.). The order of stressors was randomized for each group. The f_H transmitters were switched on using a handheld magnet 1 h before each stressor, and the stressors were applied at 1200 hours. The f_H was recorded for the 20 min before, 30 min during, and 10 min after each stressor. Blood samples to measure CORT levels induced by the stressor were taken within 3 min of workers' entering the experimental room immediately after the stressor was completed.

The birds completed their molt approximately 20 d after the last stressor was administered. Basal 24-h f_H and CORT measurements were taken from the same eight birds 2 wk after molt had completed. The above protocol was then repeated, using the same four stressors in a different order. The f_H and CORT responses to acute stressors in European starlings have been shown to be similar when the stressors were repeated in the same individual in the same physiological state (Romero and Remage-Healey 2000; Nephew et al. 2001).

Blood samples were centrifuged at 400 *g*, and plasma was removed and stored at −20°C to be used for CORT analysis. CORT concentrations were estimated using the radioimmunoassay previously described by Wingfield et al. (1992).

Statistical Analysis

JMP (ver. 5.1, SAS Institute) was used for all statistical analyses. Our statistical analysis for the calibration experiment followed Green et al. (2001). Least squares regressions were used to determine the relationship between heart rate and energy expenditure (kJ/d) for each individual. A general linear model was used to compare the slopes of the relationship between f_H and energy expenditure under stressed and unstressed conditions. We used a repeated-measures experimental design to analyze changes in f_H, CORT, and energy expenditure by comparing changes in the same individuals during and after molt. Repeated-measures (rm) ANOVA is robust to deviations from normal distribution unless deviations result in heterogeneous variances (Quinn 2002). Thus, we used the Shapiro-Wilk *W* goodness-of-fit test to test for normal distributions and Levene's test to test for homogenous variances in the data. For all variables, either distributions were normal or deviations from normality did not result in heterogeneous variances. Consequently, two-way rmANOVAs were used to compare changes in f_H, CORT, and energy expenditure during molt and nonmolt. We also used a Fisher's protective least squares difference (PLSD) test for posthoc analysis of rmANOVAs. We analyzed the f_H response to each stressor in two ways. First, we used a one-way rmANOVA on the f_H data collected before and during each stressor to determine whether the stressor altered f_H from baseline levels. Second, we used a two-way rmANOVA to compare the peak f_H response (maximum f_H during stress minus baseline f_H) to each stressor between molt and nonmolt conditions. Mass data were also normally distributed (Shapiro-Wilk *W* test: $W = 0.96$, $P = 0.68$); thus, we used a paired *t*-test to analyze changes in M_B when birds were molting and not molting. All means are represented ± SE.

Results

Calibration of Heart Rate versus Oxygen Consumption (Respirometry)

The f_H and energy expenditure (kJ/d) were highly correlated for each individual (Table 1), and the best relationship across all individuals was best described by the linear fit: $\dot{V}O_2 = 0.306f_H - 3.38$ (Fig. 1; mean $r^2 = 0.798$). This relationship was not improved by $\log_e$ transformation of the data (mean $r^2 = 0.736$). We used an ANCOVA to determine whether sex affected the relationship between f_H and energy expenditure in starlings, because female starlings weigh substantially less than males

Table 1: Individual regression equations of heart rate and energy expenditure (kJ/d) of the eight birds used in the calibration experiment

Sex	a	b	r^2	P
F	−14.115	.322	.973	<.0001
F	17.256	.264	.955	<.0001
F	−78.876	.411	.906	<.0001
F	−2.631	.222	.797	<.0001
M	2.02	.321	.832	<.0001
M	−11.148	.351	.936	<.0001
M	−23.201	.387	.819	<.0001
M	−14.817	.369	.835	<.0001

Note. The best relationship across all individuals was $\dot{V}o_2 = 0.306f_H - 3.38$. There were 14 (seven during the day and seven during the night) data points/individual used to compute each regression equation. a = y-intercept; b = slope of the linear regression.

(Feare 1984). However, males and females showed similar relationships between f_H and energy expenditure (Table 1; $F_{1,108} = 2.03$, $P = 0.16$). Furthermore, the slopes of the relationships between f_H and energy expenditure under stressed and unstressed conditions were similar ($F_{1,108} = 0.15$, $P = 0.70$).

Heart Rate Changes in Molting Starlings

Males and females did not have significantly different responses in any f_H and energy expenditure comparisons (all $P > 0.05$) except for the single intruder and only during nonmolt. A single intruder increased f_H and energy expenditure during nonmolt in both sexes, but females had a greater response ($F_{1,49} = 2.03$, $P < 0.01$). These results were consistent with previous data indicating that sex differences in captive starlings in either CORT release or f_H are rare (Romero and Remage-Healey 2000; Nephew et al. 2003). Consequently, all eight starlings were pooled for analyses.

Baseline f_H throughout a 24-h period was significantly higher during molt ($F_{1,14} = 45.4$, $P < 0.0001$; Fig. 2*A*), yet activity levels were similar between molting and nonmolting birds ($F_{1,14} = 0.8$, $P > 0.39$; Fig. 2*B*). Figure 3 shows the f_H for the 20 min before and 30 min during each stressor trial, and each stressor elicited an increase in f_H over baseline levels (measured 20 min before administering the stressor) during both physiological states (all $P < 0.01$) except for the single-intruder stressor during molt ($F_{1,49} = 0.69$, $P = 0.94$). However, the stress-induced increase in f_H was significantly greater for all stressors when the birds were not molting (all $P < 0.01$), except for the intruder stressor with five birds ($F_{1,49} = 1.22$, $P = 0.16$).

The peak f_H response to each stressor was higher during nonmolt ($F_{1,14} = 12.45$, $P > 0.003$; Fig. 4). Restraint elicited the greatest peak f_H response during nonmolt (Fisher's PLSD $P < 0.05$). During molt, all stressors increased the peak f_H response over the single-intruder stressor (Fisher's PLSD $P < 0.05$). In addition, recovery time was significantly different in molting and nonmolting birds ($F_{3,39} = 13.4$, $P < 0.001$; Fig. 5). The f_H recovered the slowest for the restraint stressor during nonmolt (Fisher's PLSD $P < 0.001$) and the auditory stressor during molt (Fisher's PLSD $P < 0.05$).

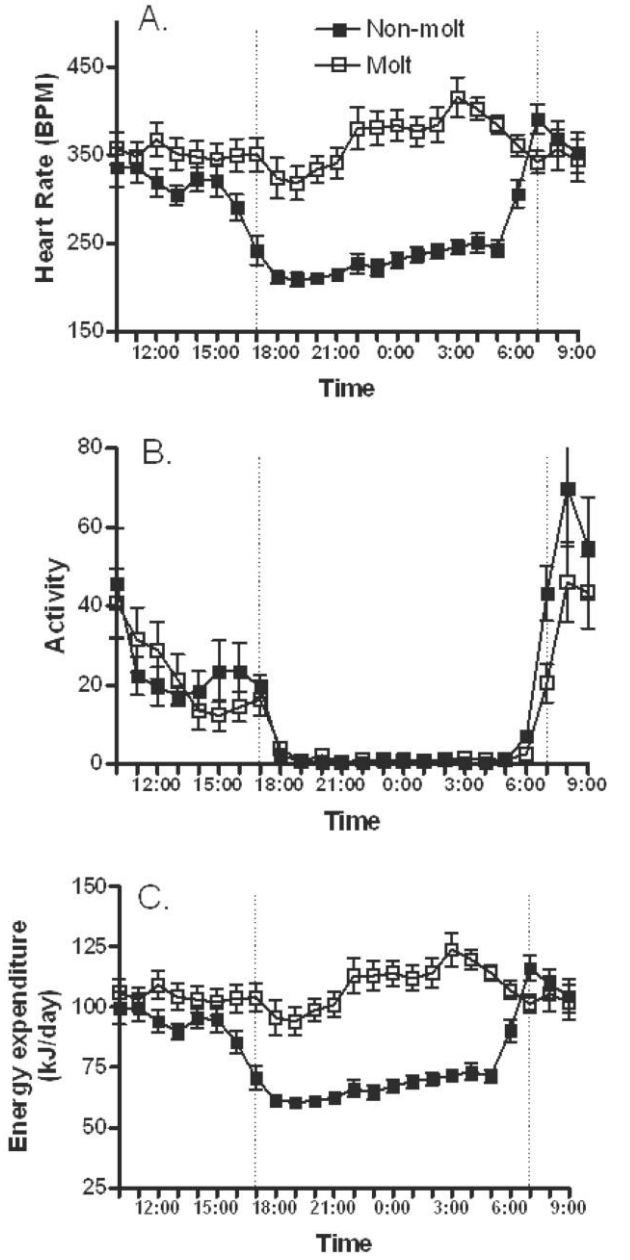

Figure 2. *A*, Mean heart rate (beats per minute [BPM]; ±SE). *B*, Mean activity (±SE). *C*, Mean energy expenditure (kJ/d; ±SE, calculated from f_H, using the relationship presented in Fig. 1) over a 24-h period for eight European starlings during molt and at 3 wk after completing molt (nonmolt). Data were collected as 5-min averages and averaged over 60-min bins.

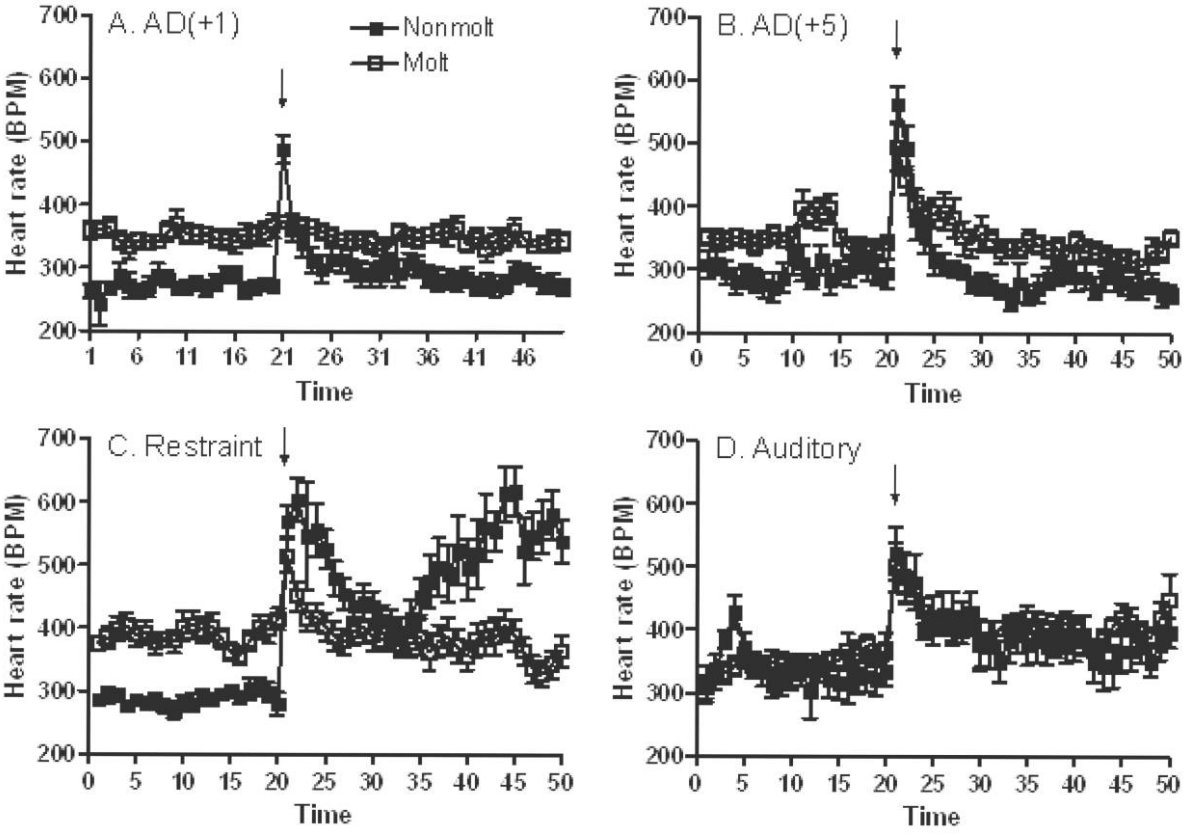

Figure 3. Mean heart rate (beats per minute [BPM]; ±SE) of molting and nonmolting starlings before and during introduction of one bird (*A*), introduction of five birds (*B*), restraint (*C*), and auditory stressor (*D*). The first 20 min show baseline values; these are followed by the onset of the stressor (*arrow*). The next 30 min are during the stressor. AD = acute density stressor in which one or five birds were introduced into the resident's cage.

Energetic Changes in Molting Starlings

Baseline 24-h energy expenditure ($F_{1,14} = 46.27$, $P < 0.0001$) was significantly higher when the birds were molting than when they were not molting. Starlings expended 32% more energy over 24 h during molt than during nonmolt (Fig. 6). Although molting starlings did not expend more energy during the daytime ($F_{1,14} = 1.76$, $P = 0.21$), energy expenditure at night was significantly greater during molt (60% over nonmolt; $F_{1,14} = 81.69$, $P < 0.0001$).

Energy expenditure increased during all stressors (all $P < 0.05$) except the single-intruder stressor during molt ($F_{1,49} = 0.69$, $P = 0.94$). However, only the auditory stressor during molt and the restraint stressor during nonmolt significantly increased energy expenditure over the normal daily energy expenditure ($F_{1,14} = 7.78$, $P = 0.015$, Fisher's PLSD post hoc test for restraint and auditory stressors, all $P < 0.05$). Restraint elicited the greatest increase in energy expenditure in nonmolting birds (Fisher's PLSD $P < 0.0001$), which ultimately resulted in a 64% ± 7.6% increase in daytime energy expenditure (Fig. 7*A*). The auditory stressor increased daily energy expenditure by 19% ± 6.8% during both molt and nonmolt (Fig. 7*B*), the AD + 1 stressor increased daily energy expenditure by 8.5% ± 3.4% in nonmolting birds, and all other stressors did not increase energy expenditure above 3% ± 6.0%.

CORT Changes in Molting Starlings

CORT concentrations did not vary between the sexes ($F_{1,14} = 0.11$, $P = 0.74$). Furthermore, body mass (M_B) has been shown to change during molt in several passerine species (Murphy 1996), but M_B in our starlings did not vary during or after molt (molt: 86 g ± 2.1 g; nonmolt: 89 g ± 2.1 g; paired *t*-test, $t = 1.34$, df = 7, $P = 0.22$). Consequently, all eight starlings were pooled for analyses as well.

CORT levels induced by each stressor were lower when the birds were molting ($F_{3,52} = 3.36$, $P < 0.02$; Fig. 8). During molt, not one acute density stressor elicited a CORT response as levels were similar to baseline levels (Fisher's PLSD $P > 0.3$), whereas restraint and the auditory stressor elicited a moderate CORT response during molt (Fisher's PLSD $P < 0.05$). In contrast, five intruders, restraint, and auditory stressors each elicited a CORT response (Fisher's PLSD $P < 0.05$); only the single-intruder stressor with one bird failed to increase CORT above baseline concentrations (Fisher's PLSD $P = 0.27$).

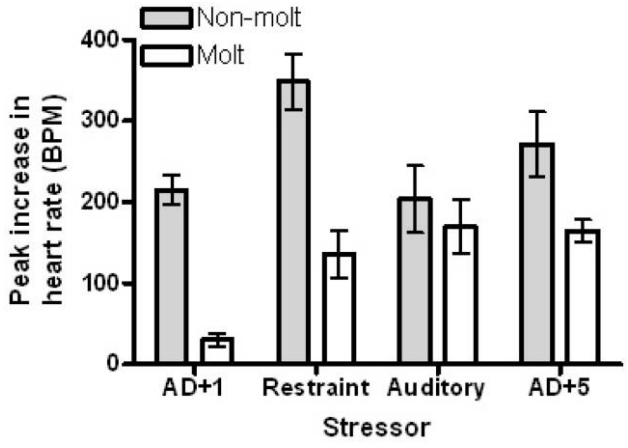

Figure 4. Stress-induced peak increase in f_H (beats per minute [BPM]; ± SE) for molting and nonmolting birds. The f_H was normalized by subtracting the mean baseline resting f_H measured 20 min before the onset of each stressor from the maximum f_H during each stressor. AD = acute density stressor in which one or five birds were introduced into the resident's cage.

Discussion

Previous studies have shown that CORT release is lowest during molt in many passerines. We hypothesized that in addition to CORT, f_H and energy expenditure also would be altered during molt, which suggests that the entire stress response changes during this energetically expensive life-history stage. We found that baseline and stress-induced f_H change in a manner parallel to changes in CORT, thus supporting our hypothesis. Also, birds expended more energy, especially at night, during molt. Finally, our results show that the energetic cost to different 30-min stressors differed with stressor and physiological state. Restraint during nonmolt caused the greatest increase in daytime energy expenditure.

CORT

Stress-induced CORT was significantly lower during molt, which supports the results of previous studies conducted in free-living animals (Romero 2002). Although this seasonal variation is lost in certain species kept in captivity, such as Lapland longspurs (*Calcarius lapponicus*; Romero et al. 1998), captive European starlings consistently show a significant reduction in baseline and stress-induced CORT during molt (Romero and Remage-Healey 2000). The physiological relevance of CORT modulation during molt is still under investigation but has been postulated to result from an avoidance of CORT's catabolic functions during protein deposition (Romero et al. 2005).

The CORT responses to the different stressors during both physiological stages were similar to those reported in earlier work on captive starlings (Nephew and Romero 2003; Nephew et al. 2003). The restraint and auditory stressors elicited a CORT release in both physiological states, but the five-intruder stressor stimulated CORT release only when birds were not molting. This suggests that the birds altered their perception of whether an increase in conspecific density was a stressor. Starlings congregate in large flocks outside of the breeding season (Feare 1984), and the reduced CORT response to increases in density during molt may represent a physiological correlate to this seasonal change in behavior (Dickens et al. 2006). This type of gradient response has been observed in other species, especially rats, where the more intense the stressor, the greater the CORT response (Armario et al. 1986; Romero et al. 1995).

Heart Rate and Energy Expenditure

The use of f_H to estimate energy expenditure has been validated in several avian species and found to be as accurate as the DLW technique (Nolet et al. 1992; Bevan et al. 1994, 1995; Green et al. 2001). In our starlings, f_H and energy expenditure were highly correlated in each individual, and gender did not alter that relationship. The relationship between f_H and energy expenditure should be consistent during molt as long as cardiac stroke volume and oxygen pulse do not change or change systematically (Butler 1993). Evidence in white-crowned sparrows (*Zonotrichia leuchphrys gambelii*; Chilgren and Degraw 1977; Murphy and King 1992) and Harris's sparrows (*Zonotrichia querula*; Degraw and Kern 1985) indicates that blood volume increases during molt, but it is unknown whether cardiac stroke volume increases. Regardless, even an unsystematic increase in cardiac stroke volume and oxygen pulse, driven by the increased blood volume, would simply mean that our estimates are conservative, because given Fick's equation, energy expenditure would be greater than our predicted values. Furthermore, molt did not alter the relationship between f_H and metabolic rate in macaroni penguins (*Eudyptes chrysolophus*; Green et al. 2004). Changes in body mass also can affect the relationship between f_H and

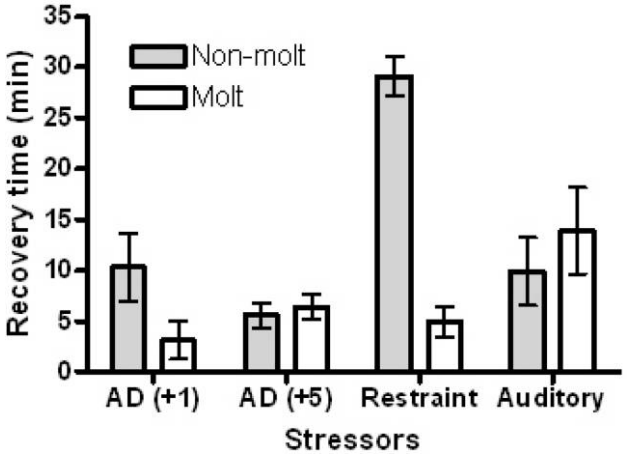

Figure 5. Mean recovery time (± SE) during the stressor for molting and nonmolting birds. Recovery time was defined as time for the heart rate to return to within 1 SE of the baseline value averaged over 20 min before the stressor. AD = acute density stressor in which one or five birds were introduced into the resident's cage.

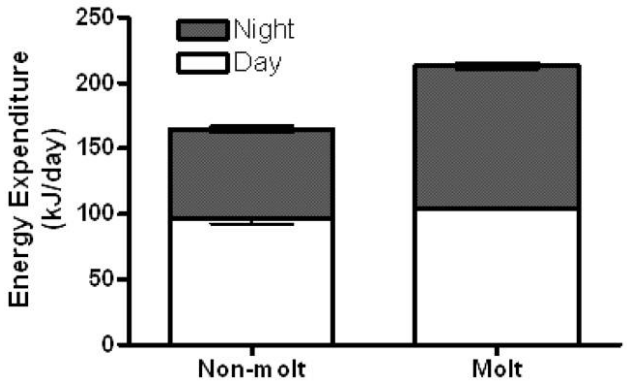

Figure 6. Baseline energy expenditure (±SE, calculated from f_H using the relationship presented in Fig. 1) during molt and nonmolt. Day (lights on) is represented by the white bars, and night (lights off) is represented by the black bars.

metabolic rate (Froget et al. 2001). Passerines are typically at the nadir of their body mass at the onset of molt and then gradually increase their mass as molt progresses (see Murphy 1996). However, mass did not change during molt in our starlings. Thus, it seems reasonable to use f_H to estimate energy expenditure during both physiological states.

Baseline energy expenditure measured over a 24-h period was significantly higher during molt, yet there were no differences in the birds' activity levels. Several studies have used basal metabolic rate (BMR) before and during molt to estimate the cost of feather synthesis (e.g., Dietz et al. 1992; Klaassen 1995), and most have found that BMR is elevated during molt in passerine species (Lindström et al. 1993). For example, Klaassen (1995) found that the highest BMR during the year is during molt for European stonechats (*Saxicola torquata rubicula*) and for East African stonechats (*Saxicola torquata axillaries*). Other studies have shown increases in $\dot{V}O_2$ during molt of 9%–111% greater than premolt levels (King 1981; Lindström et al. 1993). Because researchers measured BMR using different techniques in different species and at different times during molt, there is a wide range in values (reviewed in Murphy 1996). Furthermore, Murphy and King (1992) used the nutritional requirements of molt to determine that white-crowned sparrows increased their energy expenditure 3%–20% during molt, with the highest energy expenditure corresponding to the peak in molt. Similarly, our starlings expended an estimated 32% more energy at peak molt than during nonmolt. Interestingly, daytime energy expenditure increased by 8%, whereas nighttime energy expenditure increased by 60%. Body composition and peripheral blood flow are altered during molt (e.g., Chilgren and Degraw 1977; Holt 1992). For example, during molt there is increased blood flow at the skin because the follicle of a new, growing feather is engorged with blood, whereas that of an old (nongrowing) feather is not. These changes may explain the increased energy expenditure that we detected during molt. Furthermore, Murphy and King (1990, 1991) suggested that energy required to store proteins for feather production may be expended at times when birds are not feeding, such as overnight, which may explain why we observed a greater increase in energy expenditure at night than during the day. Our results show that molt increases energy expenditure, and our estimates using f_H to calculate energy expenditure are similar to estimates of molt costs in other passerines using other methods. However, more work is needed to understand the physiological changes occurring during the day and at night in molting birds.

Heart Rate and Stress

The f_H response to three of the four different stressors used in this study was damped when birds were molting. A previous study in nonmolting starlings showed that of these four stressors, restraint elicited the greatest increase in f_H (Nephew et al. 2003). Therefore, we predicted that restraint would cause the greatest

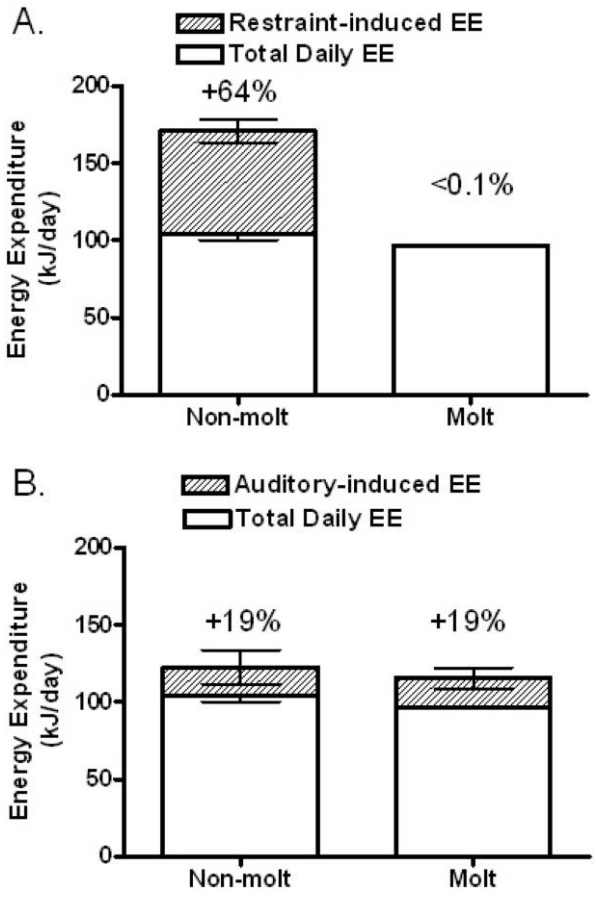

Figure 7. Increase in the energy expenditure (EE) rate during restraint (as calculated from f_H using the relationship presented in Fig. 1). The white bars indicate the mean energy expenditure (±SE) during the daytime (lights on) for molting and nonmolting starlings. The bars with hatching represent the percentage of the normal daytime energy expenditure (see Fig. 6) that was additionally expended during the 30-min restraint. Values were calculated by subtracting the baseline energy expenditure before restraint from the energy expenditure during restraint.

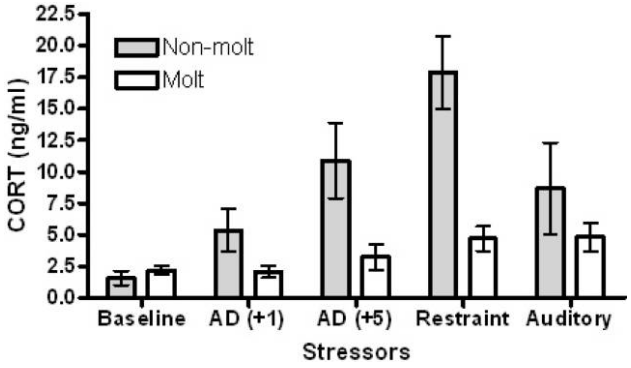

Figure 8. Mean corticosterone (CORT) concentrations (ng/mL ± SE) during the stressor for molting and nonmolting birds subjected to four different stressors. AD = acute density stressor in which one or five birds were introduced into the resident's cage.

increase in f_H during both physiological states. However, although restraint induced the greatest increase in f_H in nonmolting birds, all but the single-intruder stressor induced approximately equivalent increases in f_H during molt. Previous studies have shown that an animal's physiological state can alter its response to the same stressor. For example, female willow ptarmigan display freezing behavior in response to a threat (human approach to the nest), with nonincubating females showing tachycardia and incubating females showing bradycardia (Steen et al. 1988). The recovery time after the onset of each stressor also differed between molting and nonmolting birds. The longest recovery time was elicited by the auditory stressor during molt, but f_H essentially never returned to baseline during restraint in nonmolting birds. Taken together, these results provide further support for the hypothesis that the birds respond differently to stressors during these two physiological states.

Strikingly, the f_H response induced by each stressor was lower during molt despite the higher baseline f_H. This change in f_H response to acute stress mimicked the attenuated stress-induced CORT levels found in molting starlings. We used tachycardia to estimate the strength of the first wave of the stress response. A parallel study using heart rate variability analysis showed that f_H is under sympathetic control during acute stress in European starlings; thus, f_H can be used to estimate the sympathetic response to an acute stressor in this species (Cyr et al., forthcoming). Therefore, our results indicate that the first and second waves of the stress response are altered during molt in a parallel manner.

It is unclear why stress-induced f_H is lower during molt, but changes in the f_H response to stress could potentially have fitness consequences. For example, attenuations in the f_H response to stress could suggest an inability to mount an adequate stress response, which may dramatically reduce the animal's ability to survive (Sapolsky et al. 2000).

The f_H response to individual acute stressors did not reflect the total energetic costs of responding to those stressors. The auditory stressor during molt caused a significant increase in daytime energy expenditure, but the restraint stressor in molting birds and both intruder stressors during molt and nonmolt did not increase daytime energy expenditure. The lack of a significant energetic cost to mounting a response to many of these stressors resulted from a transitory tachycardia with a rapid return to baseline f_H. The response simply did not last long enough to expend significant energy. In contrast, the restraint stressor resulted in a remarkable increase in daytime energy expenditure in nonmolting birds (64% over baseline energy expenditure), indicating a substantial energetic cost to this 30-min stressor. There is some evidence, however, that birds may lower their nighttime energy expenditure to compensate for the increased energy expended during restraint (Buttemer et al. 1991). Further study is needed to understand the relevance of the energetic cost to restraint, especially given that restraint is a common technique used in both captive and field studies of birds.

Conclusion

Molt dramatically impacts the overall stress response. The f_H and energy expenditure, not just CORT release, are altered during molt. The f_H response to stress (first wave of the stress response) and the CORT response to stress (representing the second wave of the stress response) were reduced during molt. The result is a general damping of physiological and endocrine stress pathways. The consequences of this damping are not clear but could potentially result in a diminished capacity to adequately respond to stressors. Therefore, passerines may be at a greater physiological risk when faced with an acute stressor during molt. Furthermore, the 32% increase in daily energy expenditure during molt demonstrates an energetic cost to molt, which may reduce the overall energy available for a stress response.

Finally, we showed that our starlings did not increase their daytime energy expenditure during most acute stressors. However, the auditory stressor significantly increased daily energy expenditure during molt. Moreover, the 30-min restraint stressor, a stressor commonly used in both laboratory and field studies, caused a significant increase in daytime energy expenditure in nonmolting starlings. This suggests a potential cost to mounting a stress response to restraint that might require a trade-off with other physiological or behavioral responses. However, the other stressors, arguably more representative of those encountered in nature, did not provoke a significant response or significant energy expenditure. These results suggest that the type of stressor administered affects the extent to which experimental results can be extrapolated to stress responses of birds in the wild.

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