Heart Rate and Heart-Rate Variability Responses to Acute and Chronic Stress in a Wild-Caught Passerine Bird

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ABSTRACT

The cardiovascular-stress response has been studied extensively in laboratory animals but has been poorly studied in naturally selected species. We determined the relative roles of the sympathetic nervous system (SNS) and the parasympathetic nervous system (PNS) in regulating stress-induced changes in heart rate (HR) in wild-caught European starlings (*Sturnus vulgaris*). In both heart-rate variability (HRV) analysis and receptor blockade (atropine and propranolol) experiments, baseline HR was controlled predominantly by the PNS, whereas the increase in HR resulting from acute restraint stress was controlled predominantly by the SNS. These results indicate similar cardiac control of baseline and acute-stress-induced HR in wild-caught starlings and mammals. We further investigated HR responses during chronic stress. Driven primarily by changes in PNS regulation, baseline HR increased during the day but decreased at night. In addition, elevated HRs during acute restraint stress were attenuated throughout chronic stress and were accompanied by decreased HRV. This suggested that increased SNS drive elevated HR, but the attenuated HR response combined with resistance to the SNS blocker propranolol suggested that the sympathetic signal was less effective during chronic stress. Overall, chronic stress in wild-caught starlings elicited profound changes in cardiac function that were primarily regulated by changes in the PNS.

Introduction

All vertebrates mount a stress response to noxious stimuli that is regulated by the sympathetic-adrenal-medulla axis and the hypothalamic-pituitary-adrenal (HPA) axis. The cardiovascular component of the stress response to an acute (single, short-lived) stressor includes increased heart rate (HR), increased blood pressure, and diversion of blood to muscles. This is a rapid response mediated by the sympathetic-adrenal-medulla axis through the release of the catecholamines epinephrine (E) and norepinephrine (NE) and is protracted by the release of glucocorticoids, which are mediated by the HPA axis (reviewed in Sapolsky et al. 2000). These hormonal and physiological changes that constitute the stress response facilitate the animal's ability to cope with unpredictable acute stress. However, either long-term activation of the stress response or exposure to frequent stressors (i.e., chronic stress) is thought to be maladaptive and can lead to various physiological consequences (McEwen 1998). For example, cardiovascular consequences of excess catecholamines include hypertension, myocardial infarction, increased cardiac output, and arrhythmias (Rupp 1999). Several techniques for inducing chronic stress have been developed for laboratory mammals, especially rats (e.g., Kvetňanský and Mikulaj 1970; Porsolt et al. 1977; Kant et al. 1983; Willner et al. 1991), but basic regulatory mechanisms are left unresolved by these studies. Although many studies have found that chronic stress leads to increased HR (e.g., Farah et al. 2004), others have found the opposite (e.g., Bartolomucci et al. 2003). Clearly, a better understanding of the cardiovascular responses to stress is needed.

To this end, this study examined both the baseline HR and the HR response to an acute stressor in wild-caught European starlings held under normal husbandry conditions and exposed to chronic stress. The overall goals of the chronic-stress portion of the experiment were to describe the changes in baseline and acute-stress-induced HR caused by chronic stress as well as to begin to identify the mechanisms underlying those changes. Once we know how HR reacts to various treatments in the lab, we can conduct similar experiments in the field to determine how animals perceive their natural environment.

In addition to measuring HR, we used two techniques to determine the relative control of the parasympathetic nervous system (PNS) and the sympathetic nervous system (SNS) over HR in birds subjected to acute and chronic stress: (1) HR variability (HRV) and (2) blockade experiments. The first technique, HRV, is commonly used in humans and laboratory mammals to determine the source of cardiac stimulation (e.g., Grippo et al. 2006; Sgoifo et al. 2006; Chuang et al. 2007; Kimura et al. 2007; Taylor et al. 2007). This technique is based on the well-accepted theory that the distance between R waves (R-R interval) in the QRS complex of an electrocardiogram (ECG) wave varies, depending on the source of cardiac stimulation. If the PNS is the main regulator of the HR (as seen

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during basal/resting conditions), then the R-R interval is more variable than if the SNS is the main regulator of the HR (as seen during acute-stress-induced conditions; reviewed in Stauss 2003). Therefore, HRV is a useful method in mammals for determining which system (PNS or SNS) is regulating HR under different conditions. A previous study in chickens demonstrated that in birds, sympathetic stimulation decreases HRV (Kuo et al. 2001). However, this technique has not been used to determine cardiac control of HR in passerine birds. One of the goals of this study was to validate the use of HRV to determine control of HR by the PNS and the SNS in a passerine. The second technique, parasympathetic and sympathetic blockade, is also a common technique used to determine cardiac regulation (e.g., Lekas et al. 1999; Phogat and Parvizi 2007; Wang et al. 2007). For our blockade experiments, we used atropine to block the PNS and propranolol to block the SNS. By blocking one system, one can measure changes in the other system under different conditions (i.e., during stress). We used these blockade experiments not only to reveal which system changed the most during stress but also to determine whether HRV changed in the predictable way when each system was the primary regulator of HR. For example, when the PNS is blocked, the SNS becomes the primary source of cardiac stimulation, and thus HRV should decrease. Consequently, we also used the blockade experiments to validate the use of HRV in a passerine bird.

Material and Methods

Animals

European starlings (*Sturnus vulgaris*) were used as model species for our study. Starlings are an excellent species in some respects. First, they are common throughout the United States and adapt well to captivity. Second, several studies of stress have used both wild-caught and free-living starlings; thus, there is a wealth of information regarding how they respond to stress (e.g., Remage-Healey and Romero 2000; Nephew and Romero 2001, 2003; Rich and Romero 2005). Finally, starlings are able to accept heart-monitor implants and retain a wide range of motion and flight (Nephew et al. 2003).

We captured 47 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. We implanted HR transmitters into all 47 starlings (see below), and immediately after surgery, we moved the birds to an experimental room maintained at 25°C and placed them in individual cages (45 cm × 37 cm × 33 cm), where they were able to see and hear each other.

Food and water were provided ad lib. to all birds at all times. All experiments were conducted according to the Association for Assessment of Laboratory Animal Care guidelines and were approved by the Institutional Animal Care and Use Committee at Tufts University.

Heart-Rate Transmitter Implantation

For a detailed description of HR transmitter implantation, see Nephew and Romero (2003). Briefly, transmitters and all surgical instruments were sterilized using MaxiCide sterilizing and disinfecting solution (Henry Schein, Melville, NY). A combination of ketamine (30 mg/kg) and xylazine (10 mg/kg) was injected into the pectoral muscle for anesthesia. For the surgery, a 15-mm incision was made in the abdomen, and then a 5-mm incision was made in the neck and another 5-mm incision was made near the pygostyle. ECG leads surrounded by flexible polyurethane were slid through the 15-mm incision to the 5-mm incisions using a trochar and sleeve. The leads were sutured twice to the muscle and then 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 body 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 post surgery to ensure recovery.

Heart Rate, Heart-Rate Variability, and Activity

HR, HRV, and activity data were collected using Data Sciences International model TA 10EA-F20 transmitters (St. Paul, MN). The transmitters send out signals to a receiver positioned on one side of each bird's cage. The data are then transferred to a computer equipped with Dataquest Advanced Research Technology Gold 4.0 software package, which records continuous ECG signals and calculates the R-R intervals. For all HR and HRV data, ECG waves were collected continuously. Figure 1 depicts representative baseline daytime (before restraint; Fig. 1*A*) and restraint-induced ECG traces (Fig. 1*B*).

HR was collected in 10-s running bins. The HR response to injections (see below) and restraint was analyzed three ways. First, we used the trace HR, which was a continuous trace of the running HR before and after injection (or during restraint). Trace HR was taken 5 min before injection and/or restraint and continued for 15 min after injection or during restraint. Second, we used the peak HR response, which was determined as the average baseline HR measured in the 5 min before injection or restraint subtracted from the maximum HR (within 60 s) after injection or during restraint. Third, we used an integrated HR measurement that was calculated by subtracting each 10-s binned HR average (until 15 min after injection or restraint) from the average baseline HR (collected 5 min immediately before the injection or restraint) and then summing those values. The integrated HR analysis provided an estimate of the total HR response to injection or restraint over 15 min.

Dataquest Advanced Research Technology Gold is a software package developed for use in rats, mice, and dogs. Consequently, we used Ponemah (Ponemah Physiology Platform, Data Sciences International, Valley View, OH) to determine the accuracy with which R-R intervals were marked for our birds

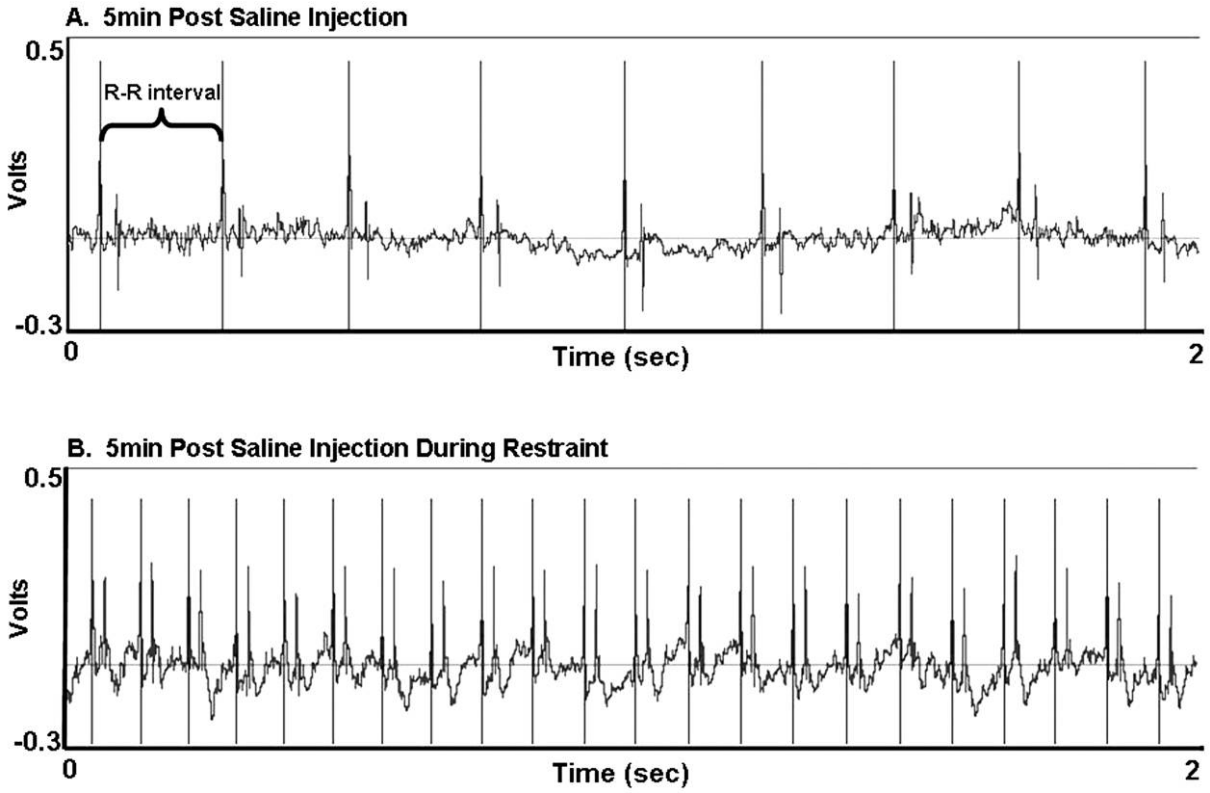

Figure 1. Electrocardiogram (ECG) traces of one representative bird. Both *A* and *B* were taken over 2 s (time on the *X*-axis) and from −0.3 to 0.5 V (voltage on the *Y*-axis). Vertical lines are marks for each R wave. *A*, Trace 5 min after injection with saline, representing baseline ECG. The bracket indicates a sample R-R interval. *B*, Trace 5 min after injection with saline and during restraint, representing acute-stress-induced ECG.

(R-R intervals are shown in Fig. 1*A*). Incorrect marks were present in all traces, although the percentage was highly variable between animals and between measurement periods. The incorrect marks were fixed manually. HRV can be analyzed using several methods, the most common being time-domain measurements and power spectral analysis, each having advantages and disadvantages (Malpas 2002; Stauss 2003). Power spectral analysis transforms the ECG from time to frequency, which is advantageous because one can then visualize the high- and low-frequency components of the ECG. The high-frequency component is mediated by the PNS, the low-frequency component is mediated by both the SNS and the PNS, and the ratio of high to low frequency can be used to determine the control of HR by the PNS and the SNS (Stauss 2003). However, a continuous ECG wave is necessary to transform the data for power spectral analysis, and a continuous signal was often difficult to obtain for our starlings. For example, electrical activity from the birds' very large pectoral muscles occasionally blocked the ECG signal. Therefore, the ECG signal was not always continuous, and we were unable to use power spectral analyses for HRV determination. Instead, we used a time-domain analysis, which has been shown to be equally effective (Kleiger et al. 1995).

For our time-domain analyses, we measured R-R intervals (in ms; see Fig. 1 for an example of an R-R interval). We used an average of 425 R-R intervals for each HRV determination. The number of measurements used did not alter any HRV comparison between groups (see "Statistics"). For each treatment, we averaged R-R intervals into 10-s running means and then subtracted that running mean from each individual R-R interval within that 10-s domain to calculate residual values. Consequently, each residual measured the difference in milliseconds between the individual R-R interval and the mean R-R interval. The greater the difference (i.e., the greater the residual), the more variable were the R-R intervals. However, when the HR increased, the residuals naturally decreased simply because there was less time between R-R peaks, meaning that the difference between individual and average R-R intervals must be smaller. To enable comparison of variability between periods with different HRs, residuals were divided by the original R-R interval to calculate a percent residual. In other words, we used percent residuals to measure variance between R-R intervals because R-R intervals change with HR. Because the higher the HR, the shorter the R-R interval, and because we wanted to compare HRV under baseline and acute- and chronic-stress conditions, we controlled for alterations in the amplitude of the HR by considering only the beat-to-beat variance (see Fig. 1).

Baseline HRV was calculated in the 5 min before administration of an acute restraint stressor, and restraint-stressor-

induced HRV was calculated in the first 6 min after the onset of restraint. To measure HRV caused by injections, we used ECG data from 5–6 min after injection because injections typically took 2–5 min to begin to alter HR.

Activity data are derived from the signal strength of the HR transmitter. A continuous signal is sent from the transmitter to two antennas on the receiver. When the bird moves, the angle of the signal to the two antennas changes, and each change generates a data point. Therefore, the more movement (greater activity), the more data points. Because activity data are generated from changes in angles, the values are relative, and therefore no units are given to activity level. We analyzed activity in 10-s bins.

Injections

All drugs were dissolved in saline and prepared the day of injection. Injections of 10 μL were administered intramuscularly into the pectoral muscle, and all doses were calculated for an 80-g starling, which is the average weight of our birds.

Propranolol (Sympathetic Blockade) and Atropine (Parasympathetic Blockade) Doses

Seven starlings, four males and three females, were used to determine an appropriate dose of propranolol to block the SNS. Birds were divided into one group of four and one group of three and given injections of (1) saline, (2) NE (0.11 mg/kg) followed immediately by saline, (3) E followed immediately by saline, (4) E followed immediately by 1 mg/kg of propranolol, (5) E followed immediately by 3 mg/kg of propranolol, and (6) E followed immediately by 6 mg/kg of propranolol. We used a dose of 0.33 mg/kg of E because similar doses have been shown to be effective in birds (e.g., Wideman 1999). Each group received each of the six treatments. There were two different injection orders, one for group 1 and one for group 2. Treatments were administered at least 24 h apart. HR was collected for 20 min before and for 15 min after injections. For parasympathetic blockade, we used a dose of 0.5 mg/kg of atropine, which has been shown to be highly effective at blocking PNS control over HR in birds (e.g., Kuo et al. 2001).

Parasympathetic Nervous System and Sympathetic Nervous System Control of Heart-Rate Experiment

A separate group of eight starlings, four males and four females, were used to determine the balance between parasympathetic and sympathetic control over baseline and acute-stress-induced HR. For this experiment, we had six treatments: birds were given injections of (1) 10-μL saline, (2) 3 mg/kg propranolol (determined from the dose-response experiment), (3) 0.5 mg/kg atropine, (4) saline immediately followed by restraint, (5) 3 mg/kg propranolol immediately followed by restraint, and (6) 0.5 mg/kg atropine immediately followed by restraint. Birds were divided into two groups of four, and each group received each of the six treatments in random order, with the order different for each group. Treatments were administered at least 24 h apart. Restraint consisted of placing birds in an opaque cloth bag for 15 min. HR and ECG waves were collected for 20 min before and 15 min after injections.

Chronic-Stress Protocol

Four groups of eight birds ($n = 32$) were subjected to a previously published (Rich and Romero 2005; Cyr et al. 2007) chronic-stress protocol that consisted of a rotation of four 30-min stressors spaced 1.5–2 h apart every day for 16 d. All groups contained both males and females. The stressors included a loud radio, rocking the bird's cage, tapping on the bird's cage, introduction to a novel human voice, and restraint in an opaque bag. All stressors were rotated except for the noontime stressor. We always exposed the birds to restraint at noontime for two reasons: to use an equivalent stressor with which we could compare acute-stress-induced changes in HR and HRV throughout the chronic-stress period, and to control for diel changes in the analysis of changes in acute-stress responses. HR and activity were monitored in all birds before and during the chronic-stress protocol. HR measurements were taken every other day, with half of the birds measured on one day and the other half measured on the next.

For two of the groups of birds receiving the chronic-stress protocol, we gave a 5-μL injection of saline every other day to avoid the possibility that the birds would respond to the injections as a novel stressor. These birds were then given a 10-μL injection either of saline or of 3 mg/kg propranolol (sympathetic blockade) immediately before the noontime restraint stressor on day 15. The opposite injection was administered on day 16 in a counterbalanced design such that four of the birds were given propranolol on day 15 and saline on day 16, and the four others received the opposite treatment. The experiments with these two groups of birds were completed before ECG traces were collected for HRV measurements. Consequently, we repeated the experiment in a third group of birds. In this group ($n = 8$), HRV was determined before and during the chronic-stress protocol during the day and during two periods at night: 2 h in the evening shortly after lights-out (between 1700 and 1900 hours, lights-out at 1700 hours) and 1 h at midnight (0000–0100 hours). HRV was also measured just before and during restraint before and throughout the chronic-stress period as well as in response to the saline and propranolol injections on days 15 and 16.

For a fourth group of eight birds (four males and four females), HR was monitored before, during, and after the 16 d of chronic stress (including measurements during the day, at night, and in response to restraint) in order to measure changes in HR after the completion of the chronic-stress period while avoiding the potentially confounding effects of the propranolol injections. In addition, activity was measured over a 24-h period before chronic stress and immediately after the completion of the chronic-stress protocol in these eight (uninjected) birds. Results from all four groups were combined for those periods and treatments that overlapped.

Statistics

SAS (ver. 9.1) was used for statistical analyses. We used a repeated-measures experimental design to analyze changes in HR and HRV by comparing changes in the same individuals before and after injections. Repeated-measures ANOVA is a parametric test that 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 all variables. For all variables, either distributions were normal or deviations from normality did not result in heterogeneous variances. Therefore, we used repeated-measures ANOVAs to compare changes in HR and HRV caused by our treatments. Fisher's protective least squares difference (PLSD) test was used for all post hoc analyses. We used two-way repeated-measures ANOVAs to test whether males and females responded differently to the treatments, and this was not the case for any treatment (all $P > 0.1$). Consequently, males and females were pooled for all analyses. We also used two-way repeated-measures ANOVAs to test whether the number of percent-residual data points altered the comparison of HRV among treatments, and this was not the case for any treatment (all $P > 0.05$). Group order of injection did not affect HR or HRV in any analysis (two-way repeated-measures ANOVA: all $P > 0.05$). Furthermore, some experiments were performed over separate trials, but the different trials did not affect HR or HRV (two-way repeated-measures ANOVA: all $P > 0.05$). Because our acute-stress experiment showed that HRV significantly decreased during restraint in starlings (see "Cardiac Regulation during Acute Restraint Stress, No Chronic Stress"), we used a one-tailed paired t-test to compare restraint-induced HRV with baseline HRV on specific days before and during the chronic-stress protocol to determine whether restraint continues to reduce HRV during chronic stress.

Results

Dose-Response Curve for Propranolol

HR was significantly different between treatment groups ($F_{40,1,783} = 3.07$, $P < 0.001$). Peak HR ($F_{5,36} = 3.11$, $P = 0.02$; Fig. 2*A*), along with integrated HR ($F_{5,35} = 2.79$, $P = 0.03$; Fig. 2*B*), also differed between groups. In our birds, saline injection did not significantly alter HR. In fact, the integrated HR response to saline injection was very low, suggesting that saline injection is not a strong stressor. However, HR was significantly higher after NE and E injections than after saline injection (Fisher's PLSD: $P = 0.020$ and $P < 0.02$, respectively). Our injections of propranolol resulted in an inverted U-shaped dose-response curve, in which the middle dose (3 mg/kg) caused the greatest decrease in integrated HR and successfully blocked the increase in integrated HR caused by E injection (Fisher's PLSD: $P < 0.02$). Some of the integrated HR responses were negative because the HR after injection was lower than the baseline HR before injection.

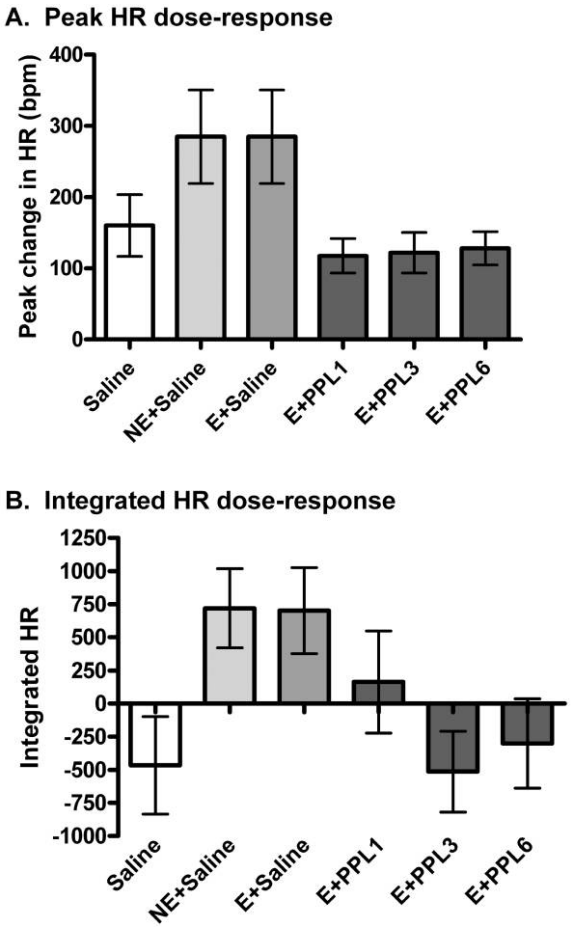

Figure 2. Dose-response curve for propranolol. Injections included (1) saline, (2) NE + saline = norepinephrine (0.11 mg/kg) immediately followed by saline, (3) E + saline = epinephrine immediately followed by saline, (4) E + PPL1 = E immediately followed by 1 mg/kg of propranolol, (5) E + PPL3 = E immediately followed by 3 mg/kg of propranolol, and (6) E + PPL6 = E immediately followed by 6 mg/kg of propranolol. All E injections were 0.33 mg/kg. *A*, Mean peak heart rate (HR ± SE) caused by injection, which was calculated by subtracting the maximum HR after injection from the average baseline HR taken 20 min before injection. *B*, Mean integrated HR (±SE) during injection, which was calculated by subtracting each HR value (until 15 min after injection) from the average baseline HR (collected 20 min before the injection) and then summing those values.

Baseline Cardiac Regulation, No Chronic Stress

HR was significantly different between birds injected with saline, propranolol, and atropine ($F_{16,2,349} = 25.5$, $P < 0.0001$; Fig. 3*A*). Atropine injection caused the greatest increase in baseline HR, and this increase in HR began to deviate from saline injection after about 2 min. Treatments also altered integrated HR ($F_{2,17} = 48.35$, $P < 0.0001$). Atropine significantly increased integrated HR over both saline and propranolol injections (Fisher's PLSD: $P < 0.001$; Fig. 3*B*), whereas integrated HR was similar between the saline and propranolol treatments (Fisher's PLSD: $P = 0.98$). Furthermore, HRV was altered by treatment ($F_{2,17} = 14.6$, $P < 0.001$). HRV was significantly lower after atropine injection that after saline injection (Fisher's PLSD:

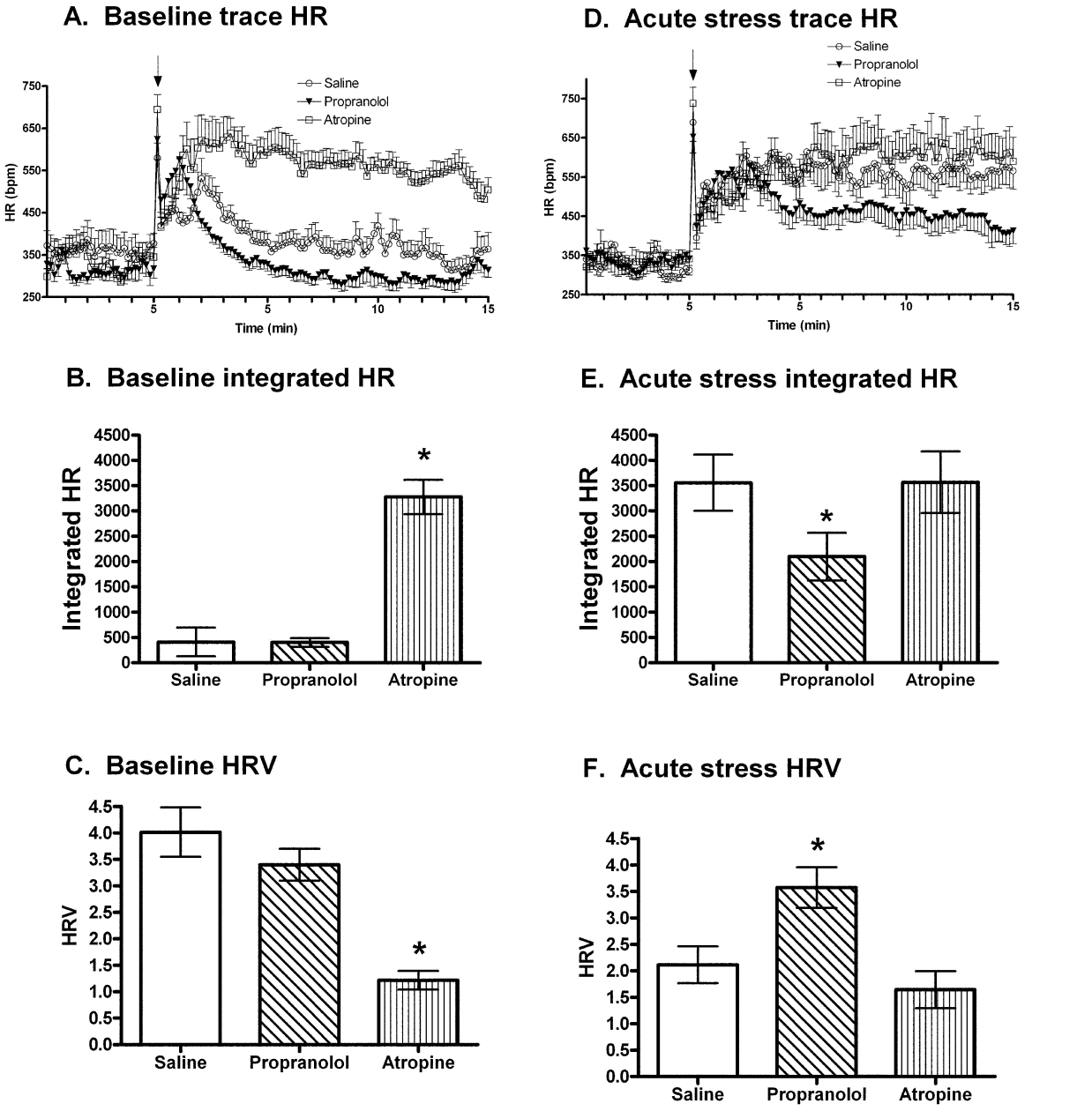

Figure 3. Heart-rate (HR) and HR variability (HRV) responses to injections of (1) saline, (2) propranolol (3 mg/kg), and (3) atropine (0.5 mg/kg). Asterisks indicate significant difference from responses to saline injection, and arrows indicate injection points. *A–C* represent baseline response because the birds were left alone after injections. *A*, Mean baseline HR (beats per minute [bpm] ± SE), taken for 5 min before and 15 min after injections. *B*, Mean baseline integrated HR (±SE) over 15 min after injections. *C*, Mean baseline HRV (±SE) measured as percent residual, taken 5–10 min after injections. *D–F* represent acute-stress-induced response because the birds were restrained for 15 min in an opaque bag immediately after injections. *D*, Mean acute-stress-induced HR (bpm ± SE), taken for 5 min before and 15 min after injections. *E*, Mean acute-stress-induced integrated HR (±SE) over 15 min after injections. *F*, Mean acute-stress-induced HRV (±SE) measured as percent residual, taken 5–10 min after injections.

$P < 0.001$; Fig. 3*C*), and it was similar in the saline and propranolol treatments (Fisher's PLSD: $P = 0.27$).

Cardiac Regulation during Acute Restraint Stress, No Chronic Stress

Injections of saline, propranolol, and atropine immediately before acute restraint stress altered HR ($F_{16,2,456} = 55.94$, $P < 0.0001$; Fig. 3*D*). However, in this case, propranolol injection caused the greatest change in HR and significantly decreased HR relative to saline and atropine injection. HR in propranolol-injected birds began to decrease below that in saline-injected birds about 5 min after injection (Fig. 3*D*). Integrated acute-stress-induced HR was also different between treatments ($F_{2,17} = 10.85$, $P = 0.002$; Fig. 3*E*) and was significantly lower

in the propranolol treatment, as compared with the saline control (Fisher's PLSD: $P < 0.002$; Fig. 2*E*), but integrated acute-stress-induced HR was similar in the saline and atropine treatments (Fisher's PLSD: $P = 0.98$). Interestingly, integrated HR after atropine injection under baseline conditions was similar to that in both the saline- and atropine-injection treatments under acute-stress conditions (Fig. 3*B*, 3*E*). In addition, HRV changed with treatment ($F_{2,18} = 6.77$, $P = 0.006$; Fig. 3*F*). HRV was significantly higher after propranolol injection than after saline injection (Fisher's PLSD: $P = 0.0004$; Fig. 3*F*), and it was similar in saline and atropine treatments (Fisher's PLSD, $P = 0.14$).

Baseline Cardiac Regulation during Chronic Stress

Daytime HR measured 5 min before restraint (i.e., at least 1.5 h after the previous stressor) increased throughout the chronic-stress period ($n = 32$, $F_{8,277} = 3.24$, $P = 0.002$; Fig. 4*A*) and recovered within 2 wk after the completion of the chronic-stress period (at days 34–35; Fisher's PLSD: $P = 0.14$). However, daytime HRV before restraint did not change during chronic stress ($F_{5,42} = 0.76$, $P = 0.58$; Fig. 4*B*). The birds' activity level 5 min before restraint also did not change during chronic stress ($F_{8,275} = 0.39$, $P = 0.92$; Fig. 4*C*).

Evening HR taken between 1700 and 1900 hours (immediately after lights-out) was reduced during chronic stress ($F_{4,69} = 3.62$, $P = 0.01$; Fig. 5*A*). Similarly, nighttime HR taken at midnight (0000–0100 hours) was reduced during chronic stress ($F_{4,73} = 4.04$, $P = 0.005$; Fig. 5*B*). Moreover, in the subset of eight birds in which midnight HR was measured before, each day during, and 3 wk after the completion of the chronic-stress period, HR remained lower than the pre-chronic-stress levels for the duration of the experiment ($F_{168,1,336} = 1.57$, $P < 0.0001$; Fig. 5*B*). In contrast, early-morning HR (0500–0600 hours, before lights-on; $F_{4,71} = 2.34$, $P = 0.06$) and morning HR (0800–0900 hours, shortly after lights-on; $F_{4,63} = 1.63$, $P = 0.18$) did not change throughout the chronic-stress period (data not shown). Interestingly, HRV did not change in any of these time periods during chronic stress (evening: $F_{4,30} = 1.0$, $P = 0.419$; Fig. 5*C*; midnight: $F_{4,30} = 1.0$, $P = 0.420$; Fig. 5*D*; early morning: $F_{4,25} = 2.39$, $P = 0.078$; data not shown; morning: $F_{4,25} = 2.45$, $P = 0.072$; data not shown).

Activity measured over a 24-h period was significantly different after the chronic-stress protocol than before the protocol ($F_{23,336} = 1.92$, $P = 0.007$; Fig. 6). The major difference was that chronically stressed birds became more active immediately before lights-on than unstressed birds.

Cardiac Regulation after Acute Restraint during Chronic Stress

Restraint-induced HR increases were significantly attenuated during chronic stress ($F_{96,3,465} = 2.38$, $P < 0.0001$; Fig. 7*A*) but recovered about 2 wk after the completion of the chronic stress. Peak HR response ($F_{8,277} = 3.20$, $P = .002$; Fig. 7*B*) and integrated HR ($F_{3,117} = 11.79$, $P < 0.0001$; Fig. 7*C*) decreased throughout the chronic-stress period. Propranolol injections on

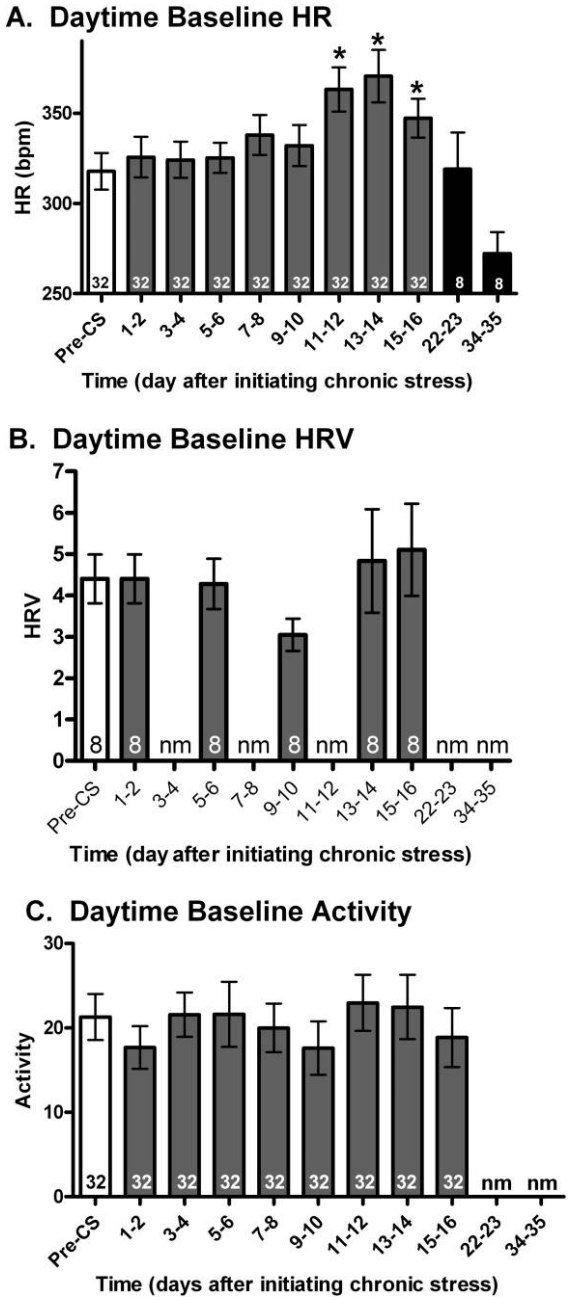

Figure 4. Daytime measurements of heart rate (HR), HR variability (HRV), and activity taken immediately before restraint before (pre-CS; *white bars*), during (days 1–16; *gray bars*), and after (after day 22; *black bars*) chronic-stress treatment. Because measurements were taken every other day, with half of the birds measured on one day and the other half measured the next, means are grouped in 2-d increments. Sample sizes are indicated within bars. Asterisks indicate significant differences from pre-CS values, as indicated by post hoc analyses. Spaces were added in *B* and *C* so that the graphs line up, and these spaces are labeled *nm* (not measured). *A*, Mean baseline HR (beats per minute [bpm] ± SE) 5 min before restraint before and during chronic stress ($n = 32$) and after chronic stress ($n = 8$). *B*, Mean daytime baseline HRV (±SE), measured as percent residual, before and during chronic stress ($n = 8$). *C*, Mean daytime baseline activity levels (arbitrary units ± SE) before and during chronic stress ($n = 32$).

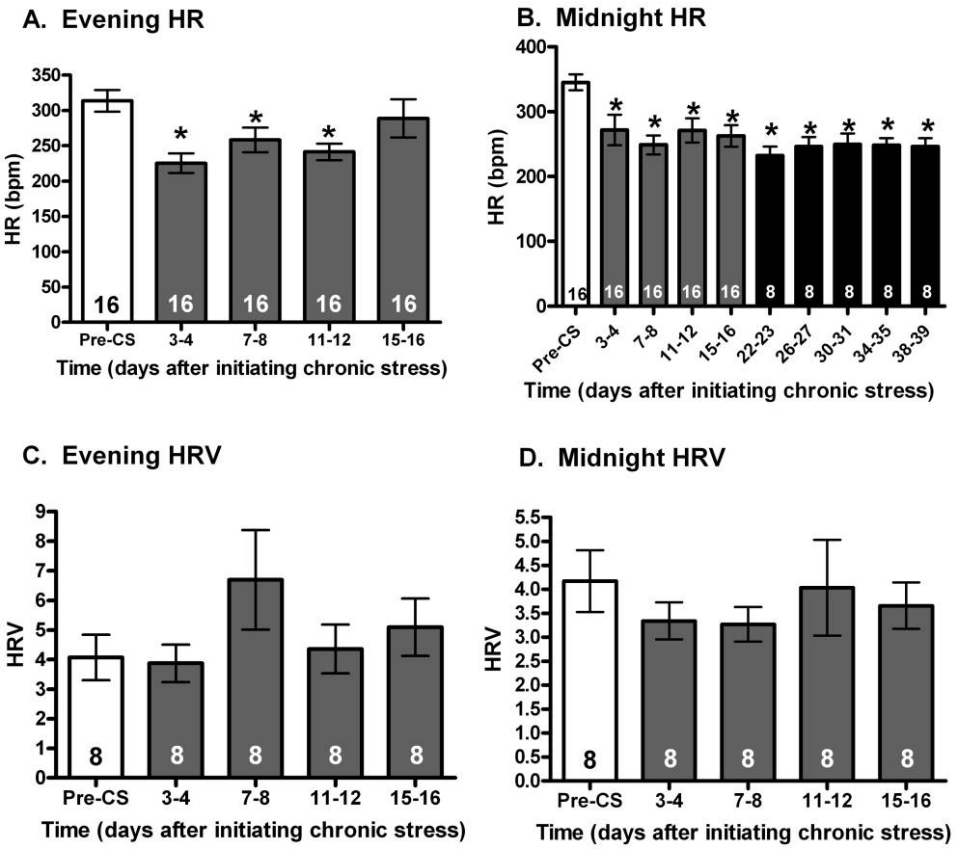

Figure 5. Nighttime heart rate (HR) and HR variability (HRV) before (pre-CS; *white bars*), during (*gray bars*), and after (*black bars*) chronic stress. Because measurements were taken every other day, with half of the birds measured on one day and the other half measured the next, means are grouped in 2-d increments. Sample sizes are indicated within bars. Asterisks indicate significant differences from pre-CS values, as indicated by post hoc analyses. *A*, Evening HR (beats per minute [bpm] ± SE), taken after lights-out (1700–1900 hours, $n = 16$). *B*, Midnight HR (0000–0100 hours, $n = 16$). *C*, Evening HRV, measured as percent residual ($n = 8$). *D*, Midnight HRV, measured as percent residuals ($n = 8$).

days 15–16 decreased HR compared with saline injections on those days, but not significantly (Fisher's PLSD: $P = 0.25$). Furthermore, restraint-induced HRV was lower than baseline HRV taken immediately before restraint during the pre-chronic-stress period (paired *t*-test: $t = -2.30$, $P = 0.03$) as well as on days 13–14 (paired *t*-test: $t = -2.45$, $P = 0.02$) and 15–16 (paired *t*-test: $t = -2.24$, $P = 0.03$) of the chronic-stress protocol (Fig. 7*D*).

Discussion

Chronic stress has been shown to increase cardiac disease in humans and laboratory mammals (McEwen 1998; Rupp 1999). Understanding the changes in HR regulation during stress in wild animals will be important in interpreting the consequences of stress in many free-living species. To our knowledge, this study is the first to demonstrate changes in HR and HRV during acute and chronic stress in a wild-caught bird, providing insight into the cardiovascular-stress responses of nondomesticated avian species. Our results indicate that the HR and HRV responses of wild-caught starlings to acute stress are similar to those in mammalian models (see Pare and Glavin 1986; Stauss 2003), but the results of our chronic-stress experiment indicate that there are differences between wild-caught starlings and mammals in their HR and HRV responses to chronic exposure to unpredictable stressors (e.g., Grippo et al. 2003; Lucini et al. 2005).

The process of adjusting to captivity could have affected the physiological responses to our stressors (e.g., Marra et al. 1995). If this were the case, we would expect HR to change over time. HR did change throughout the chronic-stress protocol, but many of these changes recovered after the completion of the protocol (e.g., daytime baseline HR and the HR response to restraint). Therefore, changes in HR were probably due to our chronic-stress protocol and not simply an artifact of captivity. There is also evidence that the physiological responses to our chronic-stress protocol may reflect responses of free-living starlings. For example, our chronic-stress protocol caused a similar reduction in plasma glucocorticoid concentrations in wild-caught (Rich and Romero 2005; Cyr et al. 2007) and free-living (Cyr and Romero 2007) starlings. However, future studies should investigate the cardiovascular stress response in free-living animals to gain a better understanding of how wild an-

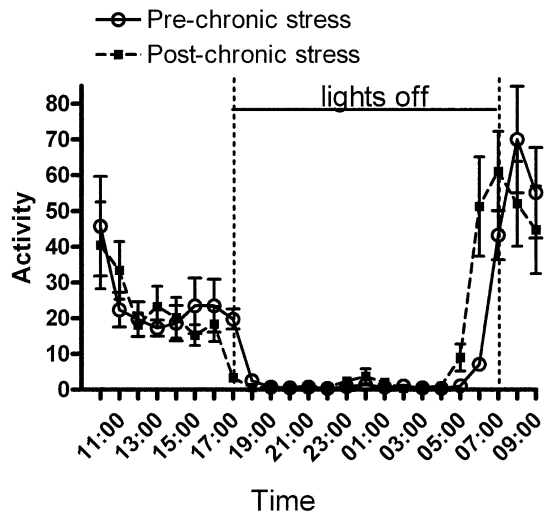

Figure 6. Mean activity levels over a 24-h period before (*solid line, circles*) and immediately after chronic stress (*dashed line, squares*).

imals living under natural conditions respond to chronic unpredictable stress.

Heart-Rate Variability as a Measure of Baseline Heart-Rate Control

HRV is a common technique used in clinical and biomedical studies to evaluate cardiac autonomic control. For example, Hayano et al. (1991) found that atropine augmented HR and attenuated HRV in normal human subjects, indicating a shift from parasympathetic control to sympathetic control. Therefore, atropine's effects on both HR and HRV lead to similar conclusions. In our starlings, we employed a similar strategy in which atropine was used to block the normal parasympathetic control and thereby expose the underlying sympathetic drive. As in the previous biomedical studies, atropine injection into our starlings significantly elevated HR and decreased HRV. These results show that atropine successfully blocked the PNS and that after PNS blockade, the control of HR shifted to the SNS.

In addition to blocking the PNS with atropine, we also used propranolol to block the SNS. Sympathetic control of HR is mediated by E and NE binding to β-adrenoceptors on the heart, and propranolol blocks these receptors (Smith et al. 2000). First, we determined the appropriate dose of propranolol for our starlings by injecting varying amounts of propranolol immediately before injecting a dose of E that is known to significantly increase HR. These propranolol injections resulted in an inverted U-shaped dose response in which the middle concentration of 3 mg/kg significantly attenuated the tachycardia caused by E injection. Previous studies in birds, including mynas (*Acridotheres*, a genus of tropical starlings), have also shown a dose-dependent HR response to propranolol (Singh et al. 1991), whereby a dose of 3 mg/kg of propranolol effectively blocked sympathetic control of HR. It thus appears that β-adrenoceptors regulate SNS control of HR in birds as well as mammals. In addition, propranolol did not significantly alter either baseline HR or baseline HRV compared with saline controls, again suggesting that the SNS plays a diminished role over baseline HR. Our results demonstrate that baseline HR in European starlings is predominantly controlled by the PNS, which is similar to findings in mammals.

Control of Acute-Stress-Induced Heart Rate

In contrast to baseline HR, sympathetic blockade with propranolol significantly decreased HR during acute restraint stress, suggesting β-adrenergic mediated sympathetic control of acute-stress-induced HR. To expand this observation, we blocked the PNS with atropine during acute stress, but parasympathetic blockade failed to significantly alter HR compared with saline controls. These results suggest a limited role of the PNS over acute-stress-induced HR. In birds, previous studies using β blockade were carried out under baseline rather than acute-stress conditions (e.g., Singh et al. 1991; Kuo et al. 2001). However, several mammalian studies have demonstrated attenuated HR after propranolol injection during acute stress (Toivonen et al. 1992; Ballard-Croft and Horton 2002). Interestingly, we found that stress-induced HRs after both saline and atropine injections were similar to baseline HR after atropine injection (see Figs. 3*B*, 3*E*), suggesting that atropine increased baseline HR to acute-stress-induced levels.

Sympathetic blockade has been shown to increase HRV in mammals (e.g., Pagani et al. 1986; Kuwahara et al. 1994; Moguilevski et al. 1996; Alipov et al. 2005). In our birds, sympathetic blockade using propranolol also increased acute-stress-induced HRV, indicating a shift from sympathetic to parasympathetic control after blockade. In contrast, the parasympathetic blockade atropine did not alter stress-induced HRV compared with saline control, suggesting that PNS activity over stress-induced HR is not significant. Overall, our results show that acute-stress-induced HR in European starlings is largely under sympathetic control.

Baseline Heart Rate during Chronic Stress

Daytime baseline HR increased during chronic stress. Several studies have reported increased synthesis and storage of catecholamines during chronic stress, which is thought to allow the animal to respond appropriately to an unknown or more severe stressor if one is presented (McCarty and Stone 1984). However, we found no difference in daytime baseline HRV throughout chronic stress, which suggests that sympathetic activity did not increase. Instead, the HRV results suggest that the PNS remained the dominant controller of baseline HR but that the magnitude of parasympathetic activity decreased with chronic stress. Little is known about the importance of parasympathetic withdrawal; however, studies using power spectral analysis of HRV have shown that humans subjected to a psychological stress (Delaney and Brodie 2000) and patients suffering from cardiomyopathy (Binkley et al. 1991) experienced

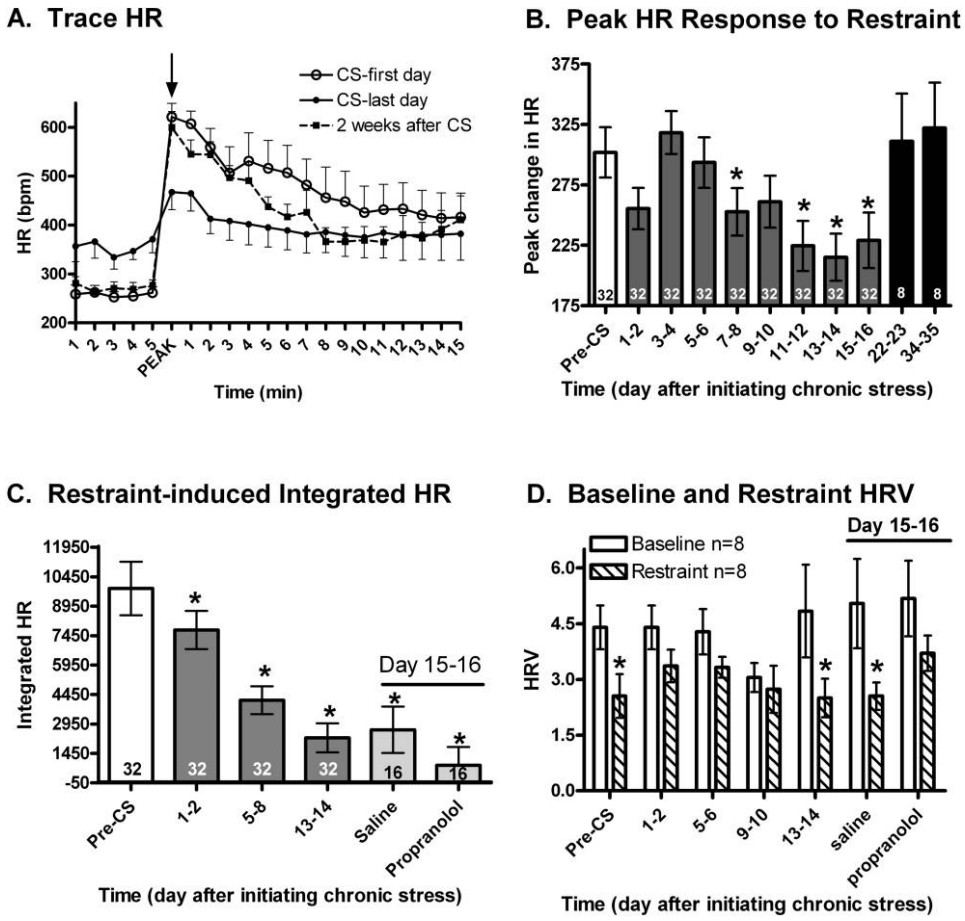

Figure 7. Acute-stress-induced heart rate (HR) and HR variability (HRV) comparisons before (pre-CS), during, and after chronic stress. *A*, Mean HR (beats per minute [bpm] ± SE), taken for 5 min before and 15 min during restraint on the first day (*open circles*) and the last day (*filled circles*) of the chronic-stress period as well as days 34–35 (*dashed line, black squares*; i.e., 18–19 d after the completion of the chronic-stress period). These 3 d are shown for simplicity; however, analyses in the text were performed on data collected before the chronic-stress period, every day during the chronic-stress period, and for 3 wk after the completion of the chronic-stress period. The arrow denotes the initiation of restraint. In *B* and *C*, white bars are for pre-CS values, gray bars are for values during chronic stress, and black bars are for post-CS values. Because measurements were taken every other day, with half of the birds measured on one day and the other half measured the next, means are grouped in 2-d increments. Sample sizes are indicated within bars. Asterisks indicate significant differences from pre-CS values, as indicated by post hoc analyses. *B*, Mean peak change in HR in response to restraint before, during, and after chronic stress. Change in HR computed as peak HR minus baseline HR from 5 min before restraint. *C*, Mean integrated HR (±SE) over 15 min during restraint before and during chronic stress. Integrated HR was computed as area under the curve for first 15 min of restraint. Light gray bars indicate saline and propranolol injection on days 15 and 16. *D*, Mean baseline HRV just before restraint (*white bars*) and mean restraint-induced HRV (*hatched bars*), measured as percent residual, taken 5–6 min after the start of restraint before and during chronic stress ($n = 8$). Asterisks indicate when restraint-induced HR differed significantly from baseline HRV on the same day.

parasympathetic withdrawal. In addition, parasympathetic withdrawal can occur during and after exercise, and it has been suggested that this is the cause of increased risk of sudden cardiac death after exercise (Billman and Hoskins 1989; Cole et al. 1999, 2000). More research is necessary to elucidate the physiological consequences of parasympathetic withdrawal in birds. However, it appears that parasympathetic withdrawal is a common phenomenon during times of both stress and increased energy expenditure.

HR during the morning, before and immediately after lights-on, did not change during chronic stress. Although HRV also did not change in the morning, there was a trend that HRV decreased before and immediately after lights-on during chronic stress, suggesting that there may have been a change in sympathetic tone. However, evening HR (immediately after lights-out) and midnight HR decreased significantly during the chronic-stress protocol. In fact, after the first day of the chronic-stress protocol, midnight HR decreased and remained attenuated during the remainder of the chronic-stress protocol and for at least a further 3 wk afterward. Because HR is strongly and positively correlated with oxygen consumption in European starlings (Cyr et al. 2008), the reduction in midnight HR probably indicates a concomitant reduction in basal metabolic rate during the night. Animals may decrease their nighttime HR and glucocorticoid concentrations to balance their energy budgets during chronic stress. Interestingly, this decrease in

nighttime HR occurred almost immediately after the initiation of chronic stress and many days before the increase in daytime HR.

There again appears to be a parasympathetic mechanism for decreased evening and midnight HR. Evening and midnight HRV did not change during chronic stress, indicating that there was no change in sympathetic output. This suggests that the decrease in nighttime HR resulted from an increase in parasympathetic activity. Although we would have anticipated an increase in HRV with increased parasympathetic output, that might not have been possible. The normal resting variability in beat-to-beat intervals is caused by respiratory gating of parasympathetic activity and is called "respiratory sinus arrhythmia" (Stauss 2003). Even though respiratory sinus arrhythmia is normal, most arrhythmias are dangerous; thus, we probably hit a ceiling in the interbeat variation caused by respiratory sinus arrhythmia at night. This possibility is supported by data indicating that propranolol, when injected into non–chronically stressed starlings, which presumably resulted in nearly exclusive parasympathetic activity, increased HRV only to approximately the same level (refer to Fig. 3*F*; Fig. 5*C*, 5*D*). Consequently, increased parasympathetic activity at rest (i.e., at night) might result in lower HR but not higher HRV.

Activity during Chronic Stress

Daytime baseline activity levels immediately before restraint did not change throughout the chronic-stress protocol (Fig. 4*C*). This suggests that the birds were not behaviorally anticipating the onset of restraint during the chronic-stress protocol and further suggests that the chronic-stress protocol successfully prevented birds from learning how to anticipate stressor presentation (Dallman and Bhatnagar 2001). In addition, activity increased before lights-on after the completion of chronic stress compared with before the chronic-stress period (Fig. 6). This suggests that the birds began to feed earlier when they were exposed to chronic stress and may provide the underlying mechanism for Rich and Romero's (2005) finding that starlings significantly increase their weight, to approximately 105% of their original unstressed weight, soon after the completion of the chronic-stress period. Further research is needed to gain a better understanding of nutritional and behavioral changes caused by chronic stress in birds.

Acute-Stress-Induced Heart Rate in a Chronically Stressed Bird

Chronic stress elicited a significant gradual attenuation of restraint-induced tachycardia. The attenuation parallels the observed decrease in restraint-induced plasma glucocorticoid concentrations during chronic stress in both captive (Rich and Romero 2005) and free-living (Cyr and Romero 2007) starlings. Clearly, the chronic-stress protocol induces a profound damping of both the first wave (sympathetic) and second wave (HPA) of the stress response in starlings.

The mechanism underlying the attenuated sympathetic response during chronic stress appears to be driven primarily by changes in the PNS. Many previous studies investigating the effect of chronic stress on sympathetic activity have induced chronic stress by repeated administration of a single stressor, and most of these studies found a reduction in the sympathetic response to that stressor on repetition (reviewed in Kvetňanský et al. 1984). Rotating different stressors to induce chronic stress, as was done in this study, had a different effect. The increase in HR induced by restraint, albeit attenuated, was accompanied by a decrease in HRV even at the end of the chronic-stress period, indicating that restraint increased sympathetic activity despite the reduced HR response. This would suggest that the restraint-induced increase in HR, even though the increase was attenuated, continued to be driven by increased sympathetic output. However, propranolol was unable to block the increased HR, suggesting that the increased HR was not a result of increased E or NE binding. Although these results appear contradictory, when the HRV and propranolol data are combined, they suggest that even though sympathetic output is increasing, the heart is not fully listening to the sympathetic signal. A logical mechanism would be a decrease in β-adrenoceptor numbers. Several studies have demonstrated a significant decrease in β-adrenoceptors on the heart in chronically stressed rats (reviewed in McCarty and Stone 1984; Torda et al. 1984), and a similar effect may have occurred in our birds. Consequently, if the increased sympathetic signal is not fully reaching the heart, the increased HR must result partially from a decrease in parasympathetic inhibition.

Earlier work suggested that the sympathetic response to a repeated stressor wanes because the animal becomes familiar with the stressor and thus reduces its response to that stressor in order to conserve energy (reviewed in McCarty and Stone 1984). Energy conservation may also be at the root of the changes we observed. One exposure to 30 min of restraint is energetically costly to starlings (Cyr et al. 2008), and our birds were exposed to four different stressors (including restraint) every day for 16 d. Responding to this chronic-stress protocol probably required substantial energy expenditure, and birds lost weight during the 16 d (Rich and Romero 2005).

Conclusion

Our results indicate that autonomic control of acute-stress-induced HR in wild-caught European starlings is similar to autonomic control in mammals, which suggests that the mechanisms underlying the cardiac response to acute stress are highly conserved. However, we found unexpected changes in autonomic control of HR when our birds were chronically stressed. For example, most mammalian studies have demonstrated increased HR due to elevated sympathetic tone during chronic stress (e.g., Grippo et al. 2003; Lucini et al. 2005). However, we found that chronic stress altered the parasympathetic control of baseline HR and that parasympathetic activity decreased during the day but increased at night in chronic-stress conditions. Furthermore, the reduction in acute-restraint-induced HR was due to a reduction in β-adrenoceptors and not a reduction in sympathetic activity. The attenuated HR response to acute stress

in our chronically stressed birds may indicate a mechanism to avoid deleterious effects of chronically high HR. However, this response may also have negative consequences. For example, a diminished HR response to acute stress may negatively affect the animal's ability to cope adequately with an acute stressor, such as a predator. The results of this study clearly show that chronic stress alters cardiac regulation in wild-caught European starlings; however, further study in free-living populations is needed to determine the cardiac mechanisms and consequences of chronic stress in free-living animals.

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