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Research Article

The effects of daily mitotane or diazepam treatment on the formation of chronic stress symptoms in newly captured wild house sparrows

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Wild animals brought into captivity frequently experience chronic stress and typically need a period of time to adjust to the conditions of captivity (restraint, artificial lighting, altered diet, human presence, etc.), to which they may never fully acclimate. Changes in mass, the hypothalamic–pituitary–adrenal axis and heart rate parameters have been observed over the first week in newly captive house sparrows (*Passer domesticus*). In this study, we tested the effects of two drugs, diazepam and mitotane, in preventing the chronic stress symptoms caused by captivity, compared with oil-injected control animals. Diazepam is an anxiolytic that is widely prescribed in humans and other animals and has been shown in some cases to reduce physiological stress. Mitotane is an agent that causes chemical adrenalectomy, reducing the body's capacity to produce glucocorticoid hormones. Our mitotane treatment did not cause the expected change in corticosterone concentrations. Baseline corticosterone was higher after a week in captivity regardless of the treatment group, while stress-induced corticosterone did not significantly increase above baseline after a week in captivity in any treatment group. However, mitotane treatment did have some physiological effects, as it reduced the resting heart rate and the duration of the heart rate response to a sudden noise. It also prevented the increase in nighttime activity that we observed in control animals. There was no effect of diazepam on corticosterone, resting heart rate, activity or heart rate response to a sudden noise, and no effect of either treatment on the sympathetic vs parasympathetic control of the resting heart rate. Together, these data suggest that mitotane, but not diazepam, can have a modest impact on helping house sparrows adapt to captive conditions. Easing the transition to captivity will likely make conservation efforts, such as initiating captive breeding programs, more successful.

Key words: Captivity, corticosterone, diazepam, heart rate, mitotane, stress

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Introduction

When an animal is faced with a real or perceived challenge to survival, it typically mounts a stress response, a set of conserved physiological reactions, to help maintain homeostasis and survive emergencies. The adrenomedullary response occurs within seconds, resulting in a release of epinephrine and norepinephrine that cause increased heart rate, cardiovascular tone and energy use (Sapolsky *et al*., 2000). The hypothalamic–pituitary–adrenal (HPA) axis response results in the release of glucocorticoid hormones from the adrenal cortex that mediates many systems to help direct resources towards survival (Sapolsky *et al*., 2000). The stress response is a critical system to help survive predators, famine, weather events, social stressors and many other small and large catastrophes that threaten survival and homeostasis (Romero and Wingfield, 2016). However, when stressors occur continuously or too frequently, the resulting chronic stress can cause physiological problems of its own (Romero *et al*., 2009). Because the stress response integrates many pathways (e.g. perception, adrenomedullary response, HPA response, etc.), and these different pathways interact, it can be difficult to determine how chronic stress symptoms form and how they can be prevented. In this study, we tested two drugs to determine if they could prevent or reduce chronic stress in a wild species: house sparrows (*Passer domesticus*). Diazepam acts on GABA receptors in the brain to decrease anxiety and thereby change the perception of stress (Licata and Rowlett, 2008), and mitotane acts at the adrenal cortex to reduce the body's ability to mount an HPA response (Sanders *et al*., 2018).

Chronic stress occurs when frequent stressors cause the stress response itself to become dysregulated (Romero *et al*., 2009). When a wild animal is brought into captivity, it faces many uncontrollable, unpredictable stimuli such as confinement, change in food, artificial lighting, presence of and handling by humans, etc. (reviewed in Cabezas *et al*., 2007; Morgan and Tromborg, 2007; Franceschini *et al*., 2008). These stimuli are likely perceived as stressors, and their constant presence can cause chronic stress. Captivity causes chronic stress symptoms in a variety of wild species, though the response of the HPA axis has been better studied than adrenomedullary responses (Fischer and Romero, 2019). Captivity can cause glucocorticoid concentrations to change in mammals (e.g. Terio *et al*., 2004; Cabezas *et al*., 2007; Franceschini *et al*., 2008), birds (e.g. Dickens *et al*., 2009; Adams *et al*., 2011; Cabezas *et al*., 2012; Lattin *et al*., 2012a; Fischer *et al*., 2018), reptiles (e.g. Jones and Bell, 2004) and amphibians (e.g. Narayan *et al*., 2011). Results from these studies suggest that chronic captivity stress can precipitate (but is not necessarily limited to) the following symptoms: (i) weight loss, (ii) changes in HPA axis function, (iii) increase in resting heart rate, (iv) increase in resting sympathetic nervous system activity and (v) abnormal heart rate response to a sudden noise (startle response).

The response of house sparrows to chronic captivity stress has been examined in three previous studies. Newly captive house sparrows had increased baseline corticosterone (Cort, the main glucocorticoid hormone in birds) compared with free-living birds (Lattin *et al*., 2012a; Fischer and Romero, 2016; Fischer *et al*., 2018) and had a higher resting heart rate and a moderately reduced startle response compared with long-term captives (Fischer and Romero, 2016; Fischer *et al*., 2018). In the current study, we used two drugs to help tease apart how the different parts of the stress response work together and how chronic stress might be ameliorated by blocking different parts of the response. The aim of this study was to determine whether the symptoms of chronic stress could be prevented from forming if the stress response was blocked at the level of the brain or the level of the adrenal cortex.

Benzodiazepines such as diazepam are widely prescribed as anxiolytics in both humans (Olfson *et al*., 2015) and domesticated animals (e.g. Herron *et al*., 2008). We hypothesized that diazepam's anxiolytic properties would be beneficial to newly caught wild birds. Daily injection of diazepam might allow the birds to perceive captivity conditions as less threatening. If so, we would predict that a low dose of diazepam might help reduce chronic stress symptoms. Specifically, we expected that diazepam-treated birds would have reduced baseline concentrations of plasma Cort, reduced weight loss, reduced heart rate, lower sympathetic nervous system activity and stronger response to startle compared with control birds.

In contrast, mitotane causes a temporary chemical adrenalectomy, killing the cells of the adrenal cortex and causing a reduction in Cort production (Breuner *et al*., 2000; Sanderson, 2006). In house sparrows, mitotane has been shown to be very effective at reducing stress-induced Cort (Breuner *et al*., 2000; Lattin *et al*., 2012b; Gao *et al*., 2017; Gao and Deviche, 2019). It can also reduce baseline Cort, though results are sometimes inconsistent (Breuner *et al*., 2000). By treating new captives with mitotane, we expected to see a reduction in baseline and stress-induced Cort, with further potential effects on the cardiovascular system. Cort acts permissively to help epinephrine and norepinephrine function (Sapolsky *et al*., 2000). Therefore, with less Cort, the effectiveness of epinephrine and norepinephrine would decrease. We predicted that heart rate and resting sympathetic nervous system activity would decrease with mitotane treatment. Our hypothesis was that attenuating the Cort response to captivity would help the birds adjust quicker to captive conditions.

House sparrows in the main experiment were treated daily with either diazepam or mitotane during the first week of captivity. We monitored their heart rate, heart rate variability (a metric of sympathetic nervous system activity; see section on heart rate variability analysis), response to startle and plasma Cort over the course of 1 week.

Materials and Methods

Drug validations and 1 month in captivity heart rate

Eight birds (five males, three females) were captured in Medford, MA and held in captivity for 4 weeks for the validation experiments. After this period of acclimation, the birds were fitted with heart rate transmitter harnesses (see Section 2.3). We used these birds to record the following: resting heart rate; heart rate variability; heart rate response to startle; acute effect of diazepam and saline on the Cort response to stress; and acute effect of diazepam, mitotane and oil injection on heart rate. Resting heart rate and heart rate variability were recorded (see Section 2.5) for 3 minutes every 2 hours for 3 days, while the birds were left undisturbed except for normal animal care. The heart rate response to startle was recorded by measuring the heart rate for 10 minutes then suddenly opening and closing the door of the bird room and recording the heart rate for a further 10 minutes.

The effect of diazepam on the Cort response to stress was assessed by collecting a blood sample within 3 minutes of entering the room and then injecting the birds with 0.5 mg/kg diazepam or saline. We selected a low dose of 0.5 mg/kg because diazepam at higher doses is sedative. In zebra finches, a fairly deep sedation is reached at 5 mg/kg (Prather, 2012), so we selected one order of magnitude lower dose. Birds were then kept in cloth bags for 30 minutes before taking a second blood sample. The same protocol was repeated 2 days later but the birds that had received diazepam now received saline and vice versa. Plasma Cort was analysed as below. We then determined the acute effect of diazepam, peanut oil and mitotane injection on heart rate. The heart rate was collected for 10 minutes before injection with diazepam, mitotane or oil and for 10 minutes after the experimenter had left the room (total time of disturbance <5 minutes). Diazepam was tested first, followed by peanut oil and then mitotane, with at least 1 day between injections. Because even a single injection of mitotane can cause long-term effects (Breuner *et al.*, 2000), mitotane was injected last. We treated the animals daily with subcutaneously administered mitotane dissolved in peanut oil (100 µl of 45-mg/ml mitotane). This treatment regime has a similar effect on plasma Cort as treating every other day with intramuscular mitotane (same dosage) but results in less bruising of the pectoralis muscle (Lattin *et al.*, 2017).

In the second set of seven birds (three males, four females; also captured in Medford, MA and held in captivity for at least 4 weeks for further validation experiments), we assessed the behavioural effects of diazepam. Video recordings of the birds were taken before and after an injection of diazepam or saline. Four of the birds received diazepam injections on the first day and three received saline. Two days later, the procedure was repeated but with the treatment and control injections switched. The number of perch hops, preening bouts, visits to food and water dishes and times they wiped their beaks on the cage or perches were then scored for 10 minutes before and 10 minutes after injections.

Experimental design

House sparrows were captured in Medford, MA between 8 September and 6 November 2015. A total of 24 animals were used in the final experiments, 8 in each treatment group. Each treatment group contained four males and four females; previous experiments have failed to find sex differences in either corticosterone release (e.g. Rich and Romero, 2001; Gormally and Romero, 2018), heart rate responses (e.g. Fischer and Romero, 2016) or behavioural responses (e.g. Gormally and Romero, 2018; Gormally *et al.*, 2018) in captive house sparrows. Immediately at capture, a series of blood samples were taken for Cort analysis (see Section 2.3). The birds were fitted with a harness-mounted heart rate transmitter device within 3 hours of capture (see Section 2.4). They were then transferred to individual cages in an animal facility on a 13L:11D light cycle. Birds were randomly assigned at capture to one of three groups—oil control, diazepam or mitotane. Each day, the birds were injected subcutaneously once per day with either peanut oil (100 µl), mitotane (225-mg/kg body weight in peanut oil) or diazepam (0.5-mg/kg body weight in saline). The birds' diet was supplemented with chopped apple, as that has been shown to improve mortality rates in mitotane-treated house sparrows (Breuner *et al.*, 2000). Birds were held in captivity for 1 week. The heart rate was automatically sampled for 3 minutes every 2 hours. On Day 6, another series of blood samples were taken for Cort analysis. On Days 1 and 7 (before their daily injection), the birds' startle response was measured. The heart rate was recorded for 10 minutes before the startle. At t = 0, the door to the room was suddenly opened and closed. The heart rate was recorded for a further 10 minutes.

All experiments complied with the *Guidelines for the Use of Wild Birds in Research* (Fair *et al.*, 2010) and were approved by the Tufts Institutional Animal Care and Use Committee.

Plasma sampling and Cort analysis

On Days 0 and 6, a series of blood samples was taken. A baseline sample for Cort was collected within 3 minutes of the bird being captured or the researcher entering the bird room, which is before or just as Cort begins to rise (Romero and Reed, 2005). The birds were held in a cloth bag for 30 minutes before taking a stress-induced sample. Birds were then injected intramuscularly with 1-mg/kg dexamethasone (DEX), an artificial glucocorticoid that stimulates negative feedback (Lattin *et al.*, 2012a). Ninety minutes after DEX injection, a final blood sample was collected. For each sample, the alar vein was punctured and ~40-µl blood was collected in a heparinized capillary tube. All blood samples were stored on ice and centrifuged at ~1200 g for 8 minutes (Centrific

Model 225, Fisher Scientific, Pittsburgh, PA, USA). Plasma was removed and stored at −20°C.

We determined Cort concentrations in each sample using radioimmunoassay following Wingfield *et al.* (1992). Samples were assayed in duplicate in a single assay and assay values corrected for individual recoveries following extraction. Detectability was 0.86-ng Cort/ml plasma and intra-assay coefficient of variation was 1.8%.

Heart rate transmitter harnesses

We used the Data Sci International PhysioTel ETA-F10 model of the heart rate transmitter. These transmitters measure 19×13×6 mm and weigh 1.6 g. They transmit on an AM radio frequency to a receiver plate attached to the side of the cage. Although the transmitters are designed to be implantable, we used a harness-mounted method described in Fischer and Romero (2016). In brief, the body of the transmitter was sewn into a waterproof fabric pouch that was secured to a 3D printed base with the leads exposed. One transmitter lead was threaded under the skin from the middle of the back to the shoulder; the other was threaded from the mid-back to the hip. The base of the harness completely hid the transmitter leads that extended out of the skin. Four 3-cm lengths of 0.5-cm wide satin ribbon were sewn to the base and then sewn together at the centre of the animals' chests—two straps were passed around the neck and two were threaded under the wings so it fit snugly against the body like a backpack. Once acclimated, the birds show no behavioural effects of wearing the backpack (Fischer and Romero, 2016; Fischer *et al.*, 2016).

Heart rate, activity and heart rate variability data collection and analysis

Heart rate and activity were recorded automatically using DataScience's Acquisition program. Beginning in the evening of Day 0 (after the birds had recovered from surgery, been given their first injection and would be left undisturbed for the night) a 3-minute sample was recorded every 2 hours. The samples were discarded when the animals had been disturbed within 45 minutes of sampling (e.g. because of the caretakers, startle response sampling or moving other animals in and out of the facility). The program also records an 'activity' metric. The receiver plates contain three radio receivers within them. Any change in the relative signal strength between the receivers is interpreted as movement within the cage and is recorded as a unitless value. The activity was analysed on DataScience's Analysis software.

The heart rate data were analysed using the Ponemah P3 Plus program from DataSciences. This program detects the R wave on the heart rate trace, allows for some noise detection and allows the user to visually inspect the data to remove inappropriate markings of R waves. All data were carefully inspected for misplaced R wave detection.

Heart rate variability analysis

Heart rate variability was calculated using Ponemah P3 Plus following the methods of Cyr *et al.* (2009). In short, a time-domain analysis was run on a clean stretch of approximately 230 heartbeats for each 3-minute sampling window. The trace was visually inspected to ensure an accurate identification of R waves and individual marks were adjusted as necessary. Heart rate variability is a unitless measure adjusted for the heart rate, with high heart rate variability indicating that beats are more irregular and low heart rate variability indicating that they are more regular. Heart rate variability is a tool that allows us to differentiate between sympathetic nervous system and parasympathetic nervous system activity. A high heart rate can be due to high concentrations of epinephrine and norepinephrine (high sympathetic activity). However, the parasympathetic nervous system also regulates heart rate; a high heart rate could alternatively indicate reduced parasympathetic activity. The parasympathetic nervous system causes the heart rate to vary with every breath cycle; variation caused by the sympathetic nervous system occurs on a longer timescale (Stauss, 2003). Therefore, by comparing the beat-to-beat intervals on a short timescale, we can determine whether the heart is being regulated primarily by the parasympathetic or sympathetic nervous systems (high heart rate variability indicates more parasympathetic and less sympathetic activity; Korte *et al.*, 1999; Perini and Veicsteinas, 2003; Cyr *et al.*, 2009).

Data analysis

All statistical analyses were conducted in R version 3.1.3 (R Core Team, 2013). Linear mixed effects models were constructed using the 'lmer' function in the lme4 package (Bates *et al.*, 2014). Bird identity was included as a random effect in all analyses. We then used the 'Anova' function in the car package (Fox and Weisberg, 2011) to calculate Type II Wald F tests with Kenward–Roger adjusted degrees of freedom. We followed this with a Tukey's multiple comparison test if warranted, using the 'glht' function from the multcomp package (Hothorn *et al.*, 2008). An alpha of $P < 0.05$ was used to determine significance.

To test for the acute effect of mitotane, diazepam or oil injection on the heart rate, we measured the integrated heart rate for 15 minutes post-injection. (This is the area under the curve, representing the total number of additional heart beats above baseline that the bird experienced.) We tested the effect of treatment on the integrated heart rate. This was followed with a Tukey's multiple comparison test on finding significance.

Baseline Cort, stress-induced Cort and strength of negative feedback were analysed in separate models. The strength of negative feedback in the HPA axis was calculated as the percentage decrease from stress-induced Cort 90 minutes after a DEX injection. Baseline Cort was below the limit of detection for every bird at capture. For analysis, all Cort

values that were undetectably low were assigned the limit of detection (0.86 ng/ml). We ran models to test the effect of treatment group at capture and after 1 week of captivity. We also ran separate models looking for a change in pre- and post-captivity levels of Cort.

For activity, heart rate and heart rate variability data, we removed data points where the birds had been disturbed (e.g. because of animal care). We then averaged the values for each light and dark cycle. We first constructed linear mixed effects models to test for a circadian effect with treatment and time of day (day or night) as fixed effects. Because there were circadian effects in activity, heart rate and heart rate variability, we constructed separate models for daytime and nighttime. We created linear mixed effects models including treatment, day of captivity and their interaction, which were analysed as above. When the treatment group was significant, we followed this with a Tukey's multiple comparison test if warranted, using the 'glht' function from the multcomp package (Hothorn *et al.*, 2008). When there was a significant treatment or interaction effect, we then looked for an effect of day of captivity in each treatment group separately.

Data from the 1 month captives are shown in the figures for comparison. However, we did not run statistical models that included those data.

Results

Validations

The effect of diazepam on the Cort response to stress was analysed using birds that had been kept in captivity for 1 month (Fig. 1A). There was no difference in baseline Cort before injection with diazepam or saline [$F_{(1,7)} = 0.38$, $P = 0.6$]. There was no effect of diazepam injection on stress-induced Cort [$F_{(1,7)} = 1.91$, $P = 0.2$]. The behavioural effects of diazepam were also analysed. The number of perch hops decreased after an intramuscular injection, but there was no effect of treatment (saline or diazepam) and no interaction between pre- and post-injection behaviours and treatment [time: $F_{(1,18)} = 9.82$, $P = 0.006$; treatment: $F_{(1,18)} = 1.27$, $P = 0.3$; interaction: $F_{(1,18)} = 0.14$, $P = 0.7$; Fig. 1B]. There were no significant effects of injection of saline or diazepam on any other behaviour that we recorded (preening bouts, beak swipes and visits to food and water dishes; $P > 0.5$ for all comparisons).

We measured the heart rate response to mitotane, diazepam or oil injections (Fig. 1C). We calculated the integrated heart rate (area under the curve, representing additional beats experienced relative to baseline) from 0 to 10 minutes post-injection and found a marginally significant difference between the three treatments [$F_{(2,10.2)} = 3.27$, $P = 0.08$; Fig. 1D]. A Tukey's post hoc test found that the integrated heart rate after diazepam was higher than after mitotane ($z = 2.31$, $P = 0.05$) and marginally higher than after

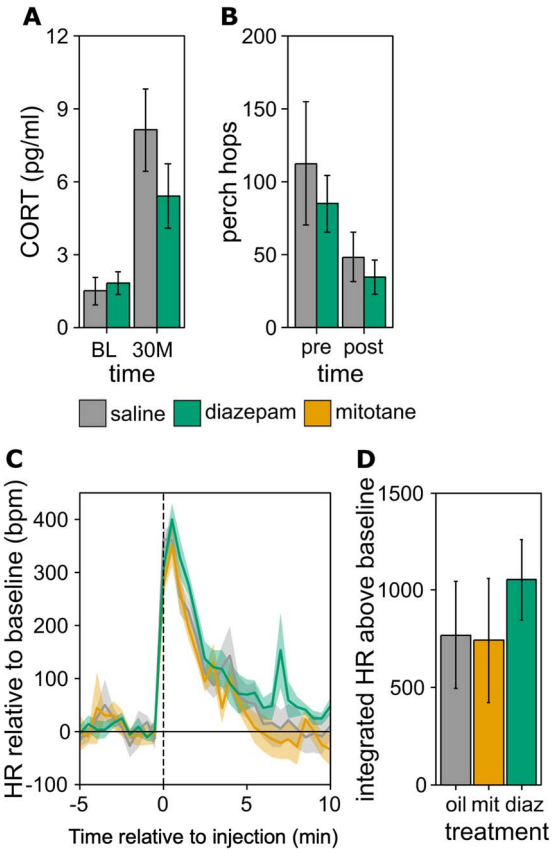

Figure 1: Validations of mitotane and diazepam. (**A**) Effect of diazepam on Cort response to stress. A total of 0.5-mg/kg diazepam or saline was injected after the baseline blood sample, then birds were held in a cloth bag for 30 minutes. (**B**) Behavioural response to diazepam injection. (**C**) Heart rate response to oil, mitotane or diazepam injection. The line and shaded areas indicate mean ± standard error. (**D**) Integrated heart rate for 10 minutes post-injection. In A, B and D, error bars represent mean ± standard error.

an oil injection ($z = 2.19$, $P = 0.07$). There was no difference in the maximum heart rate [$F_{(2,10.7)} = 1.04$, $P = 0.4$] or the time for the heart rate to return to within 1 SD of baseline [$F_{(2,10.1)} = 1.43$, $P = 0.3$] between treatments.

Weight loss after 1 week in captivity

Most birds lost weight over the course of the experiment (29 of 32). There was no effect of diazepam or mitotane treatment on weight loss [$F_{(3,28)} = 0.006$, $P = 1$]. On average, birds lost 8.79 ± 1.03% of their starting mass over the course of 1 week in captivity.

The HPA axis

At capture, the baseline levels of Cort were below the detection limit of the assay in every sample (Fig. 2). There was no effect of treatment group on stress-induced Cort at capture

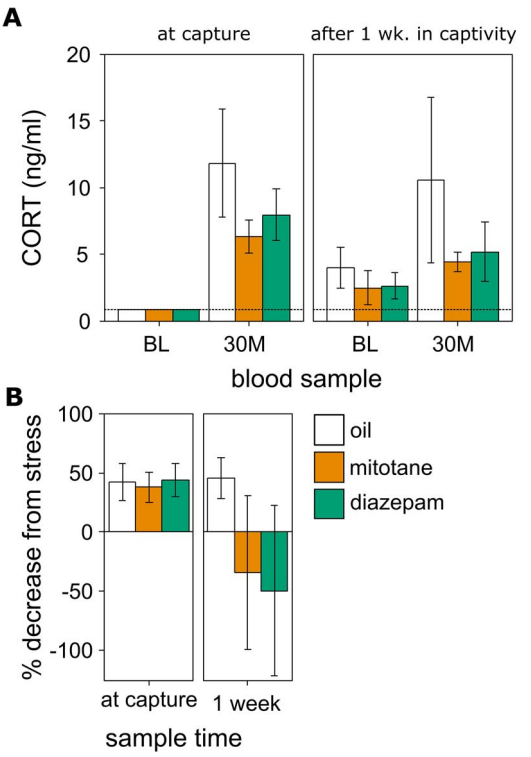

Figure 2: HPA response at capture and after 1 week of captivity. Note that at-capture samples are presented by their ultimate treatment group (oil, mitotane, diazepam), but no treatment was yet applied. (**A**) Baseline (BL) and stress-induced (30 M) plasma Cort concentrations. The dotted line indicates the limit of detection of the assay. N = 8 per treatment group. (**B**) Strength of negative feedback as the percent decrease from stress-induced concentration [% decrease = 100–100∗(Cort after DEX/Cort at 30 M)]. Higher numbers mean Cort is more reduced after the DEX test; negative numbers indicate an increase in Cort after DEX. One plasma sample was lost; therefore, $n = 8$ for each treatment group except for the diazepam at capture group, where $n = 7$. Error bars represent mean ± standard error.

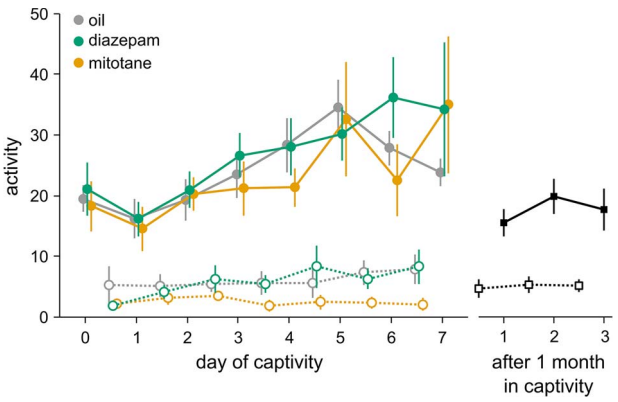

Figure 3: Activity during the first week of captivity. Activity is recorded as a unitless metric from the heart rate transmitters. Birds were fitted with transmitters at captivity and treated with oil, diazepam or mitotane once daily for the first week of captivity. Solid lines indicate activity during the lights on period; dotted lines indicate activity during the dark period. N = 8 for each treatment group. Activities from birds held for 1 month are included for visual comparison. Error bars represent mean ± standard error.

as expected, as no treatments had yet begun [$F_{(2,21)} = 1.10$, $P = 0.35$]. The 30-minute stress sample had significantly higher Cort than the baseline sample [$F_{(1,23)} = 25.36$, $P < 0.0001$]. There was no difference in the strength of negative feedback at capture between treatment groups [$F_{(2,20)} = 0.05$, $P = 0.95$].

After 1 week of captivity, there was no effect of diazepam or mitotane treatment on baseline or stress-induced Cort levels [baseline: $F_{(2,21)} = 0.43$, $P = 0.66$; stress-induced: $F_{(2,21)} = 0.77$, $P = 0.48$]. Baseline Cort was significantly higher after 1 week of captivity compared with at capture [$F_{(1,23)} = 8.93$, $P = 0.007$]. Stress-induced Cort was not different after 1 week of captivity compared with at capture [$F_{(1,23)} = 0.85$, $P = 0.37$]. At the end of the captivity period, Cort did not significantly increase after 30 minutes of restraint compared with baseline levels [$F_{(1,23)} = 3.05$, $P = 0.09$]. There was no significant difference between diazepam or mitotane treatment groups in strength of negative feedback after the captivity period [$F_{(2,21)} = 0.80$, $P = 0.46$]. There was no change in negative feedback strength in pre- vs post-captivity samples [$F_{(1,22.7)} = 2.48$, $P = 0.13$].

Activity

We analysed daytime and nighttime activities separately (Fig. 3). We ran a linear mixed model with day of captivity and treatment (with individual as a random effect) on daytime activity. We found that while activity significantly increased over the course of the first week, there was no effect of diazepam or mitotane treatment and no interaction effect [day of captivity: $F_{(1,137.3)} = 45.43$, $P < 0.00001$; treatment: $F_{(2,37.8)} = 0.07$, $P = 0.9$; interaction: $F_{(2,137.3)} = 0.38$, $P = 0.7$].

At night, there was a significant effect of night of captivity on activity level and a significant interaction effect, though no effect of diazepam or mitotane treatment [night of captivity: $F_{(1,136.3)} = 11.73$, $P = 0.001$; treatment: $F_{(2,56.1)} = 0.01$, $P = 1$; interaction: $F_{(2,136.3)} = 4.58$, $P = 0.01$]. Because of the interaction effect, we analysed each treatment group separately to look for an effect of captivity night. There was no effect of night of captivity on the mitotane group [$F_{(1,45.1)} = 1.01$, $P = 0.3$]. In the oil and diazepam groups, activity significantly increased over time [respectively: $F_{(1,44.1)} = 9.06$, $P = 0.004$; $F_{(1,47)} = 6.82$, $P = 0.01$].

Heart rate

A linear mixed effects model was run including time of day (day or night) and treatment as fixed effects and individual as a random effect. We found significant effects of treatment, time of day and their interaction on heart

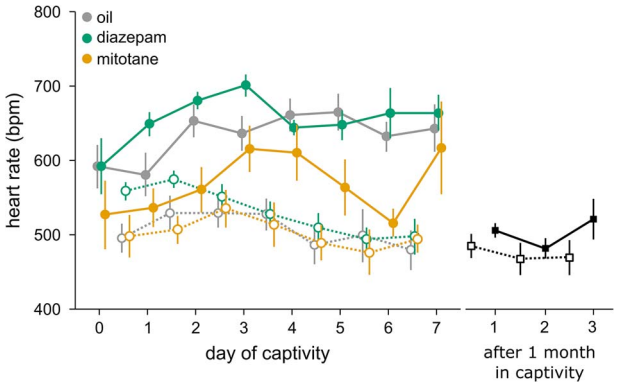

Figure 4: Heart rate during the first week of captivity. Birds were fitted with transmitters at captivity and treated with oil, diazepam or mitotane once daily for the first week of captivity. Solid lines indicate heart rate during the lights on period; dotted lines indicate heart rate during the dark period. N = 8 for each treatment group. Heart rates from birds held in captivity for 1 month and recorded over 3 days are included for visual comparison. Error bars represent mean ± standard error.

rate [Fig. 4; treatment: $F_{(2,27.8)} = 8.60$, $P = 0.001$; time of day: $F_{(1,296.2)} = 104.87$, $P < 0.00001$; interaction: $F_{(2,296.2)} = 9.79$; $P < 0.0001$]. Because of the circadian effect and the interaction effect, daytime and nighttime heart rates were then analysed separately.

For the daytime heart rate, we found significant effects of mitotane treatment and day of captivity on daytime heart rate with no interaction effect [treatment: $F_{(2,59.8)} = 3.87$, $P = 0.03$; day of captivity: $F_{(1,136.6)} = 4.55$, $P = 0.03$; interaction: $F_{(2,136.6)} = 0.48$, $P = 0.6$]. Daytime heart rate tended to increase over the course of 1 week in captivity. The heart rate in the mitotane group was lower than the oil or diazepam groups, while there was no difference between the diazepam and oil groups (Tukey's post hoc comparisons; mitotane vs oil: $z = -2.99$, $P = 0.008$; mitotane vs. diazepam: $z = 4.05$, $P < 0.001$; diazepam vs. oil: $z = 1.06$, $P = 0.5$).

At night, there were marginally significant effects of treatment and night of captivity on heart rate and a significant interaction effect [treatment: $F_{(2,33)} = 2.76$, $P = 0.08$; night of captivity: $F_{(1,133.3)} = 3.46$, $P = 0.07$; interaction: $F_{(2,133.3)} = 4.37$, $P = 0.01$]. Because of the significant interaction effect, a separate model was run for each treatment group looking for the effect of day of captivity. Nighttime heart rate significantly decreased over time in the diazepam group, but not in the oil or mitotane groups [diazepam: $F_{(1,47)} = 45.09$, $P < 0.00001$; oil: $F_{(1,44.1)} = 2.77$, $P = 0.1$; mitotane: $F_{(1,42.2)} = 0.76$, $P = 0.4$].

Heart rate variability

We found a significant effect of time of day, but no effect of diazepam or mitotane treatment or the interaction between treatment and time of day on heart rate variability

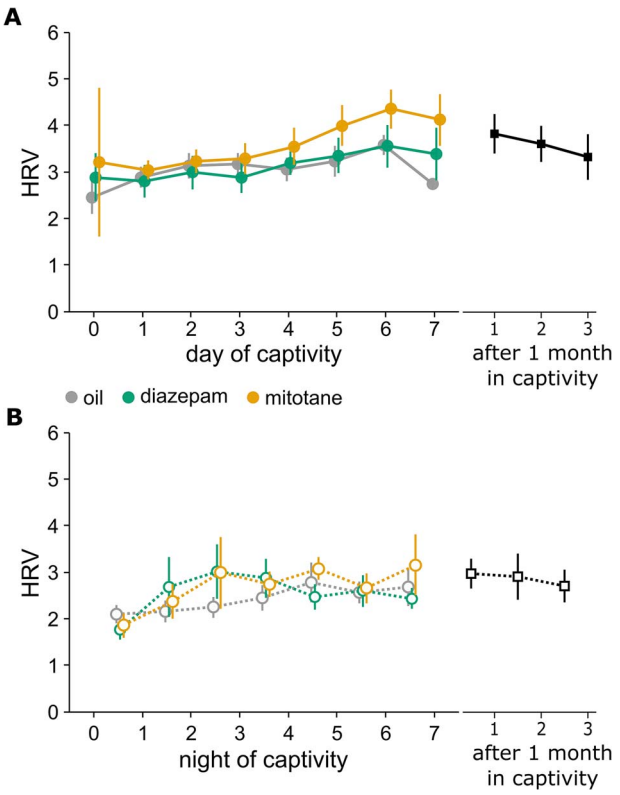

Figure 5: Heart rate variability during the first week of captivity. Birds were fitted with transmitters at captivity and treated with oil, diazepam or mitotane once daily for the first week of captivity. (**A**) Heart rate variability during the light period. (**B**) Heart rate variability during the dark period. N = 8 for each treatment group. Heart rate variability from birds held captive for 1 month are included for visual comparison. Error bars represent mean ± standard error.

[Fig. 5; treatment: $F_{(2,26.2)} = 0.57$, $P = 0.6$; time of day: $F_{(1,296.2)} = 12.16$, $P = 0.0006$; interaction: $F_{(2,296.1)} = 0.43$, $P = 0.6$]. Because of the effect of time of day, daytime and nighttime heart rates were then analysed separately.

We found no effect of treatment and only a marginally significant effect of day of captivity on the daytime heart rate variability [treatment: $F_{(2,47.0)} = 0.16$, $P = 0.9$; day of captivity: $F_{(1,136.3)} = 3.58$, $P = 0.06$; interaction: $F_{(2,136.4)} = 0.19$, $P = 0.8$]. Heart rate variability tended to increase over the first week of captivity. At night, there was a significant effect of day of captivity but not diazepam or mitotane treatment on heart rate variability [treatment: $F_{(2,36.9)} = 0.30$, $P = 0.7$; day of captivity: $F_{(1,133.4)} = 4.79$, $P = 0.03$; interaction: $F_{(2,133.4)} = 1.44$, $P = 0.2$]. The nighttime heart rate variability increased over the first week.

Startle response

The heart rate response to a sudden startle (quickly opening and shutting the bird room door) was recorded on Days 1

and 7 of captivity (Fig. 6A). From the heart rate traces, we calculated the integrated heart rate (area under the curve from t = 0 to t = 10 minutes), maximum heart rate and duration of the elevated heart rate.

We analysed the integrated heart rate on Days 1 and 7 separately to look for an effect of treatment (Fig. 6B). We found no diazepam or mitotane treatment effect on Day 1 or on Day 7 [respectively: $F_{(2,19)} = 1.59$, $P = 0.2$; $F_{(2,17)} = 1.43$, $P = 0.3$]. We also analysed each treatment group separately to look for a change in startle response between Days 1 and 7. In the oil and diazepam groups, there was no change in the integrated heart rate between the sampling days [respectively: $F_{(1,6.7)} = 0.004$, $P = 1$; $F_{(1,6.2)} = 0.45$, $P = 0.5$]. However, in the mitotane group, the integrated heart rate was lower on Day 7 than on Day 1 [$F_{(1,57)} = 26.48$, $P = 0.003$].

We conducted a similar analysis for the maximum heart rate (within 60 seconds of startle) on Days 1 and 7 separately to look for an effect of treatment (Fig. 6C). We found no diazepam or mitotane treatment effect on Day 1 or on Day 7 [respectively: $F_{(2,20)} = 2.18$, $P = 0.1$; $F_{(2,16)} = 0.49$, $P = 0.6$]. We also analysed each treatment group separately to look for a change in the maximum heart rate between Days 1 and 7. In the oil, diazepam and mitotane groups, there was no change in the maximum heart rate between the sampling days [respectively: $F_{(1,6.4)} = 0.36$, $P = 0.6$; $F_{(1,6.6)} = 0.61$, $P = 0.5$; $F_{(1,5.6)} = 0.54$, $P = 0.5$].

Finally, we analysed the duration of the startle response, defined by the time to return to within 1 SD of the baseline heart rate (Fig. 6D). We found no diazepam or mitotane treatment effect on Days 1 or on 7 [respectively: $F_{(2,20)} = 0.77$, $P = 0.5$; $F_{(2,15)} = 0.91$, $P = 0.4$]. We also analysed each treatment group separately to look for a change in the startle response duration between Days 1 and 7. In the oil and diazepam groups, there was no change in duration between the sampling days [respectively: $F_{(1,6.4)} = 0.20$, $P = 0.7$; $F_{(1,6.1)} = 0.61$, $P = 0.5$]. However, the startle response was significantly shorter in the mitotane group on Day 7 compared with Day 1 [$F_{(1,5.3)} = 7.71$, $P = 0.04$].

Discussion

Captivity can act as a powerful chronic stressor for wild animals. Unfortunately, the duration and severity of the impact of captivity are highly species-specific (Fischer and Romero, 2019). By reducing the adrenal output of Cort using mitotane or by reducing the perception of stimuli as stressful with the use of diazepam, we expected to see a reduction in the chronic stress symptoms that newly captured wild birds experienced. We expected that mitotane and/or diazepam use would help wild house sparrows acclimate to captivity more quickly. Although there were some subtle effects of both drugs, especially of mitotane, the overall effect of both drugs was marginal.

We first tested the acute effects of diazepam and mitotane on heart rate. The integrated heart rate after injection was higher after diazepam injection than mitotane injection and marginally higher than the controls (Fig. 1D). Diazepam's effects on the adrenomedullary response are inconsistent in different studies. Diazepam led to increased heart rate in rats (Conahan and Vogel, 1986; Mailliet *et al.*, 2001). However, when diazepam was given to rock partridges (*Alectoris graeca*) at a dose high enough to induce sedation, it did not affect the heart rate (Uzun *et al.*, 2006).

We also tested the acute effects of diazepam on Cort and on behaviour. Although diazepam reduces anxiety, it is not clear how the drug affects physiological stress. In some studies, a single dose of diazepam reduced the HPA response to an acute stressor, such as a forced swim test in rats (Le Fur *et al.*, 1979), being chased with a net in zebra fish (Abreu *et al.*, 2014) or a painful electric shock in humans (Roy-Byrne *et al.*, 1988). However, in this experiment, there was no difference in stress-induced Cort levels in diazepam-treated birds compared with saline injection. Similarly, in wild snow buntings (*Plectrophenax nivalis*), diazepam had no effect on stress-induced Cort in response to capturing and handling (Romero *et al.*, 1998). If diazepam reduces the stress response, it most likely does so by causing the stimulus to seem less threatening. In the case of a restraint stressor in a wild bird, low doses of diazepam probably have a little effect on the perception that the event is dangerous—the stimulus is too extreme to ignore even under the effects of the drug. Injection with either saline or diazepam caused a change in behaviour—birds were less active after injection. However, there was no difference between diazepam and saline injection.

In our main experiment, we injected newly captured birds with oil, mitotane or diazepam daily for the first week of captivity. In oil-treated birds, baseline Cort increased over the course of the first week of captivity (Fig. 2A). This is consistent with previously reported patterns over the first week of captivity in untreated or saline-treated house sparrows (Lattin *et al.*, 2012a; Fischer and Romero, 2016; Fischer *et al.*, 2018). Over the course of the first week, our oil-treated birds showed increasing daytime and nighttime activities, daytime heart rate and heart rate variability during both the day and the night. The increase in heart rate was unexpected. We had predicted that heart rate would decrease over the course of a week as the birds started to acclimate to captivity, as it did in saline-treated birds in previous studies (Fischer and Romero, 2016; Fischer *et al.*, 2018). The oil injection procedure is more difficult, however, than saline injections—we injected a higher volume and the oil itself is more viscous, requiring a higher gauge needle. We injected subcutaneously, as it was easier to inject and resulted in less muscular bruising than intramuscular injections (Lattin *et al.*, 2017). Nevertheless, daily oil injection in addition to the other stressors of captivity may have resulted in chronic stress to which the birds were unable to acclimate. To support this conclusion, resting heart rate in European starlings increased during a rotating

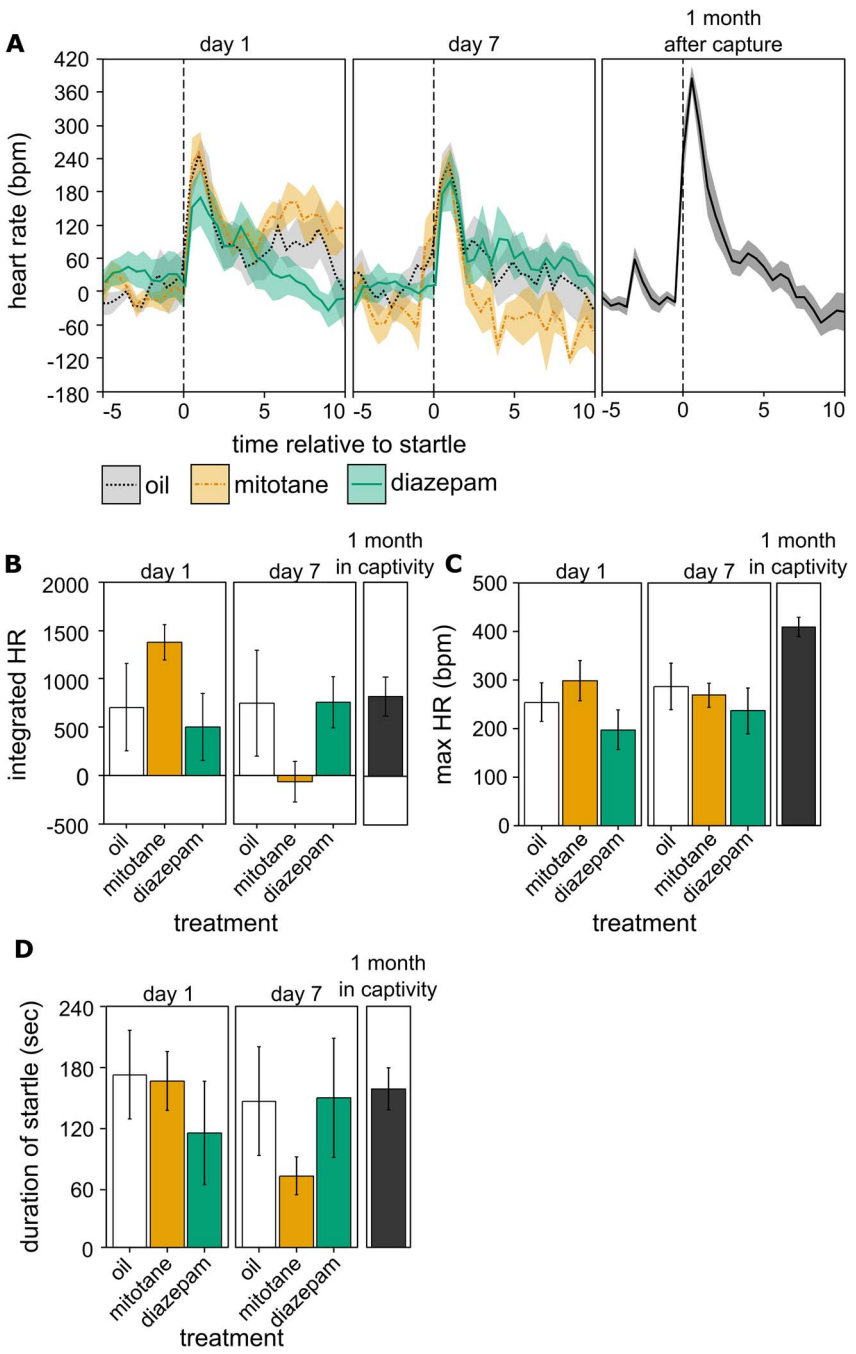

Figure 6: Heart rate response to sudden noise. On Day 1 of captivity, heart rate was sampled before and after the door to the bird room is opened and shut. Birds were held 1 week and treated with oil, mitotane or diazepam daily and a second startle response was recorded. (**A**) Heart rate trace over time relative to the baseline heart rate. The lines and shaded area indicate mean ± standard error. Note that although birds are divided by treatment group at capture, treatment had just begun. (**B**) The integrated heart rate is calculated as the area under the curve during the 10 minutes following the startle and represents the number of additional heart beats above baseline. (**C**) Maximum heart rate within 1 minute of the startle (relative to baseline). (**D**) Time for the heart rate to return to within 1 SD of the baseline heart rate. Error bars indicate mean ± standard error.

chronic stress protocol (Cyr *et al.*, 2009); the increase in heart rate may therefore indicate that our control animals were chronically stressed. In addition, the oil itself may also impact the heart rate. Dogs fed a high fat diet for >4 weeks and maintained at 150% of their initial body mass showed a markedly increased heart rate that was caused by decreased

parasympathetic control of the heart (Van Vliet *et al*., 1995). High-lipid diets can cause changes in the heart rate over the short term as well; rabbits had increased heart rate during the first 3 days of a high-fat diet (Barzel *et al*., 2014). On the other hand, it is unclear how subcutaneous oil injections would correspond to high-lipid diets. Somewhat paradoxically, however, we also saw increased heart rate variability over the course of the first week, as we had initially predicted. The increase in heart rate variability indicates that the birds were shifting to more parasympathetic control of heart rate, which indicates a reduction in chronic stress. Parasympathetic control appears to be a major regulator of heart rate in birds (Muller *et al*., 2018). Daytime heart rate variability measurements are probably unreliable, however, as the heart rate variability's interpretation relies on a resting animal. If the animal is moving around the cage during the measurement period, the variability may be the result of movement and not reflective of the rhythms of the parasympathetic and sympathetic nervous systems. However, the pattern still holds at night, when activity is very low, although nighttime activity also increased over the study period. It is possible that while the heart rate increases during the day in oil-treated birds, sympathetic activity at night decreases. In starlings facing a rotating chronic stressor, heart rate decreased at night (though heart rate variability was not affected; Cyr *et al*., 2009). One potential interpretation is that oil-treated birds reduced their sympathetic activity at night to compensate for the increased energy use fueling the increased activity.

Our mitotane treatment was not as effective in reducing Cort as expected. Mitotane selectively targets glucocorticoid-producing cells in the adrenal cortex; although to our knowledge, this has not been confirmed in birds. The death of these cells results in a severe reduction in Cort production (Sanderson, 2006). Breuner and colleagues found that within 36 hours of initiating daily intramuscular mitotane injections, baseline Cort was undetectably low and there was no stress-induced increase in Cort after 30 minutes of restraint (Breuner *et al*., 2000). The reduction of stress-induced Cort by mitotane in house sparrows was confirmed by Lattin *et al.* (2012b). Baseline Cort production after mitotane may be somewhat variable, as when Breuner *et al.* (2000) used a higher dose of mitotane, there was no difference from controls in baseline Cort. In our experiment, there was no difference in baseline or stress-induced Cort between mitotane- and oil-treated birds (Fig. 2A). However, stress-induced Cort in the oil-treated birds was low (11.9 ± 6.1 ng/ml compared with 15–30 ng/ml in untreated or saline-treated birds held in captivity for 5–7 days; Lattin *et al*., 2012a; Fischer and Romero, 2016; Fischer *et al*., 2018). There was no statistical difference between baseline and stress-induced Cort levels, even in the control animals. It is still possible that mitotane injection was effective in reducing adrenal capacity, but the unexpectedly low stress-induced Cort in the oil-treated controls prevented detecting the difference statistically.

Although our mitotane-treated birds did not show the difference in Cort concentration compared with controls that we expected to see, we did see changes in the heart rate that are consistent with reduced Cort. Daytime heart rate was lower in mitotane- than oil-treated birds (Fig. 4). Cort has permissive effects on the action of epinephrine and norepinephrine (Sapolsky *et al*., 2000). Therefore, a reduction in the heart rate is consistent with reduced Cort in the mitotane-treated birds. Mitotane-treated birds during the first week of captivity had heart rates more similar to 1-month captives than the oil-treated controls. Mitotane might therefore help reduce tachycardia in newly captured birds, which could lead to more positive health outcomes. High heart rate has been associated with death in captive birds (Domingo *et al*., 1991), so mitotane treatment at capture may have some benefits. However, mitotane itself can cause problems—if the birds' diets are not supplemented with chopped apple, mitotane can cause up to 50% mortality in newly captured birds (Breuner *et al*., 2000). The adrenomedullary response to startle was also affected by mitotane treatment; heart rate returned to baseline more quickly after a startle in mitotane-treated birds (Fig. 6D). If these birds do indeed have somewhat reduced Cort, we would expect a reduced startle response, again because of the permissive effects of Cort on epinephrine and norepinephrine. Mitotane also affected nighttime activity (Fig. 3); mitotane-treated birds were less active at night throughout the first week of captivity than oil-treated controls. Cort is associated with wakefulness, so this may be some indication that total Cort secretion was indeed lower in the mitotane group. Daytime heart rate variability was higher in mitotane-treated birds than in oil-treated controls (Fig. 5A). This could indicate higher parasympathetic and less sympathetic nervous system activity. However, as discussed above, daytime heart rate variability is probably less reliable than nighttime measurements because of the interference of activity.

The adrenomedullary impacts of mitotane treatment are evident even when the expected decrease in plasma Cort was not. This could in part be due to the snapshot nature of plasma Cort sampling. We measured plasma Cort at only two timepoints—capture and Day 6. However, we sampled heart rate and heart rate variability on a regular basis, acquiring multiple snapshots every day. If plasma Cort was lower in mitotane than oil birds during part of the week, that may have been enough to result in a marked difference in daytime heart rate. This indicates that the high heart rate we see due to chronic captivity stress may be linked to Cort production—the interaction between the arms of the stress response is important to how chronic stress develops.

The use of mitotane, however, will likely need to be assessed for each species. Not only is mitotane not effective in all species, but also it can lead to death in some individuals (Breuner *et al*., 2000). On the other hand, mitotane is an approved drug for human medicine to treat adrenocortical carcinomas (Ardolino *et al*., 2020) and has been used to treat Cushing's syndrome in humans (Tritos and Biller, 2020) and pet dogs (Sanders *et al*., 2018). Reports indicate that 5% (Puglisi *et al*., 2020) to 30% (Baudry *et al*., 2012)

of human patients discontinue therapy because of adverse side-effects. However, reproductive function is not generally affected in humans, with normal pregnancies being reported (Puglisi *et al*., 2020), and there was no effect on testis weights in house sparrows (Breuner *et al*., 2000). Reports indicate that mitotane is teratogenic (Tritos and Biller, 2020), so it is probably advisable to cease mitotane treatment several months prior to initiating long-term captive breeding programs. Critically, most human and dog studies administer mitotane for months to years (Sanders *et al*., 2018; Puglisi *et al*., 2020). Adverse effects of a few injections to ease the transition to captivity, as in this study, are not known but unlikely to be as severe as reported in the human and canine clinical literature. For example, 2 weeks of every-other-day injections in house sparrows had no impact on the mass of fat, heart, testis, pectoral muscle, spleen or whole body mass, although there was an increase in the liver mass (Lattin *et al*., 2017). In addition, the protocol used here is unlikely to be permanent; house sparrows appeared to have completely recovered 10 days after a single injection (Breuner *et al*., 2000). In conclusion, the health risks for the protocol used in this study are likely to be small and short-lived and may be completely off-set by the benefits of easing an animal's transition to captivity.

In contrast to mitotane, diazepam had little effect on the development of chronic stress symptoms. Diazepam has been shown to reduce the HPA response to acute stress in some circumstances (Le Fur *et al*., 1979; Roy-Byrne *et al*., 1988; Abreu *et al*., 2014). It has also been shown to reduce the signs of chronic stress when administered long term. For example, in a placebo-controlled double-blind trial, elderly people had reduced cortisol after 21 days of diazepam treatment (Pomara *et al*., 2005). In the present study, diazepam had no effect on Cort, heart rate parameters or activity. This is similar to findings in tree shrews (*Tupaia belangeri*) exposed long-term to social stress, where a 5-mg/kg daily oral dose of diazepam resulted in no change in cortisol, norepinephrine, weight or behaviour (Van Kampen *et al*., 2000). The conditions of captivity may be too intense for diazepam to be helpful in alleviating physiological stress. The stressors encountered during captivity are unrelenting and animals acclimating to those conditions have no reprieve from their confinement and novel surroundings. If diazepam reduces stress by changing the perception of situations to seem less stressful, it is probably less useful the more intense and ongoing those stressors are.

In conclusion, diazepam resulted in no difference in heart rate, heart rate variability, Cort or activity. It does not appear to be an effective tool for reducing chronic stress in newly caught wild birds. On the other hand, reducing adrenal output by the use of mitotane may help wild house sparrows acclimate to captivity, particularly by reducing their heart rate, more quickly to the level of fully acclimated birds. However, the repeated oil injection may be an additional chronic stressor. If mitotane is used as a tool to help birds acclimate to captivity, a single dose at captivity may be a better alternative than repeated injections. Although mitotane does have some health risks, it is likely that those risks are outweighed by the benefits of easing the transition to captivity. Reducing the stress of acclimating to captivity will aid conservation efforts by increasing the health of individuals that have to be brought into captivity for purposes such as rescuing endangered animals or initiating captive breeding programs.

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