How do wild birds adjust to captivity? Impacts on stress physiology and behavior.

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ABSTRACT

When wild animals are brought into captivity, they experience many stimuli that may be interpreted as potentially dangerous and activate the stress response, a set of hormonal pathways that are activated to maintain homeostasis in the face of harmful stimuli. If stressors are repeated or ongoing, the stress response may become dysregulated and lead to the suite of symptoms known as "chronic stress." In this dissertation, I reviewed the current literature to determine whether chronic stress decreases with time after capture, indicating adjustment to captivity. While some chronic stress symptoms frequently decrease over time (i.e. weight loss, leukocyte redistribution), other symptoms can linger for months or years in some species (i.e. elevated glucocorticoids, reproductive dysfunction). I conducted several experiments on the chronic stress of captivity in newly-captured wild house sparrows (*Passer domesticus*). I monitored glucocorticoid levels, adrenomedullary variables, and weight in house sparrows over the first 6 weeks of captivity. The birds had decreased mass up to day 35, elevated baseline glucocorticoids at day 7 that then declined, and elevated heart rate until day 20, indicating that the animals suffered chronic stress that only decreased after several weeks. To determine whether chronic captivity stress could be reduced pharmacologically, I tested the effects of 4 drugs over the first 7 days of captivity. The anxiolytic diazepam and the α-adrenergic receptor antagonist phentolamine had no effect on chronic stress. The β-adrenergic receptor propranolol prevented the increase in baseline glucocorticoids. Mitotane (which causes chemical adrenalectomy) resulted in

decreased heart rate after 1 week, even when it did not cause the expected reduction of glucocorticoids.

The hormones of stress can influence behavior. Neophobia, or the fear of novel objects, is an ecologically relevant, easily quantified behavior. While characterizing neophobia in captive wild birds, I documented a seasonal effect on food motivation in house sparrows. Finally, I analyzed the connection between the adrenomedullary response and neophobia in another passerine, the European starling (*Sturnus vulgaris*). I found no relationship between heart rate and behavior. Therefore, not every aspect of captivity (i.e. novelty) may contribute to the overstimulation of the stress response leading to chronic stress symptoms.

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CHAPTER 1: INTRODUCTION

Wild birds are frequently brought into captivity for conservation or research. Captivity eliminates some of the variability of studying wild animals in their native habitat and enables some studies that would not be possible in the field. However, the conditions of captivity can also lead to physiological changes due to chronic activation of the stress response (Morgan and Tromborg, 2007). The stress response consists of a set of systems that allow an animal to survive and maintain homeostasis during unpredictable events that are perceived as harmful or threatening (Romero and Wingfield, 2016). The adrenomedullary response – the release of epinephrine and norepinephrine – causes a rapid increase in heart rate and muscle tone (Sapolsky *et al.*, 2001). The glucocorticoid response causes a shift in resources away from growth and reproduction and towards immediate survival and maintenance of homeostasis (Sapolsky *et al.*, 2001). These systems are independently regulated but not entirely separate. For example, elevated glucocorticoids can enable epinephrine/norepinephrine to act more effectively (Sapolsky *et al.*, 2001). Although the conditions of captivity are free of the weather events, predator attacks, and food shortages that we often associate with stress, confinement, human presence, and other psychological stressors can cause continual activation of the stress response (Morgan and Tromborg, 2007). Repeated or ongoing stressors can cause an animal to experience homeostatic overload, where the stress response itself begins to cause problems (Romero *et al.*, 2009). We consider an animal to be "chronically stressed" if they display some of the set of

symptoms (e.g. weight loss, long-term immune changes, reproductive failure) associated with repeated or ongoing stressors, or if regulation of the stress response itself becomes dysfunctional.

Although captivity stress has been associated with behavioral and physiological problems in many captive wild animals, it is often assumed that many or most captive animals will adjust to captivity at some point. Indeed, many species flourish in captivity, though other species do not tolerate captivity as well (Mason, 2010). In the field of wild animal physiology (and in stress research in particular), captive wild animals are frequently used to determine the pathways of hormones and their effects on physiology. However, results of experiments are frequently different in captive animals compared to animals held in the lab (Calisi and Bently, 2009). The chronic stress of captivity may explain why physiology so often differs in lab and field studies. In Chapter 2 of this dissertation, I conducted a literature review to determine whether captivity conditions lead to long term changes in weight, glucocorticoid hormones, the adrenomedullary system, the immune system, and the reproductive system. I specifically focused on studies where wild-caught captive animals were compared to free-living animals or were repeatedly sampled as they adjusted to captivity. For some chronic stress symptoms (changes to weight or leukocyte distribution), captive animals resemble free living animals more as they adjusted to captivity over time (see sections 2.4 and 2.6). However, for some variables (changes to glucocorticoids and reproduction), many studies recorded differences between captive and wild animals even after months or years in captivity (see sections 2.5 and 2.7). Across many species, the response to captivity

was frequently affected by the time of year the study was conducted in (i.e. if the same species was measured at multiple times of year, results would vary by season). These seasonal effects were inconsistent between species. Therefore, the variability that we see between studies could be largely due to species-specific seasonal differences in sensitivity to captivity stressors.

The house sparrow (*Passer domesticus*) is a frequently used species for research into wild bird physiology. Because a great deal of research has been done in this species, there is a deep knowledge base that researchers continue to build upon (Anderson, 2006). However, the period of adjustment to captivity has not been previously documented in this species. After 5 days in captivity, house sparrows lose weight and have elevated glucocorticoids, which are symptomatic of chronic stress (Lattin, *et al.*, 2012a). However, in many studies, researchers allow animals 2 weeks to 1 month to adjust to captive conditions, under the assumption that after that period, their physiology will resemble a wild animal, or at the very least have stabilized to some "new normal" that will not afterwards change, no matter how long they are kept in captivity (e.g. Bókony *et al*., 2014; Lattin and Romero, 2014). In Chapter 3, I observed the period of adjustment to captivity in newly-captured house sparrows for the first 6 weeks of captivity. I repeatedly sampled weight and baseline glucocorticoids. I focused on the effects of captivity on the adrenomedullary system. I measured heart rate, heart rate variability, and the adrenomedullary response to a sudden noise (the startle response) repeatedly over time. To do this, I developed a new harness mounted system for using implantable heart rate transmitters in small birds that do not tolerate the surgery required for

implantation (see Chapter 4 for full description). *I hypothesized that animals would initially show symptoms of chronic stress, but these would decrease by the end of the study period.*

Captivity stress is something that wild animal physiologists and conservation biologists may want to avoid or reduce as much as possible. In Chapters 4 and 5, I discuss several possible pharmacological techniques to reduce chronic captivity stress. In Chapter 4, I present results of an experiment in house sparrows to determine whether blocking the hormones of the adrenomedullary system will improve chronic stress symptoms. *I hypothesized that temporarily blocking α- and/or β-adrenergic receptors would reduce chronic stress symptoms during the first week of captivity.* In Chapter 5, I present the results of using two other pharmacological agents to potentially reduce chronic stress: mitotane (which causes a chemical adrenalectomy and a reduction in glucocorticoids) and diazepam (an anxiolytic and sedative). *I hypothesized that mitotane would reduce the weight loss and adrenomedullary symptoms of chronic stress by reducing circulating glucocorticoids, and diazepam would reduce chronic stress symptoms by decreasing the perception of stress.*

Captive wild animals are also frequently used in behavioral studies. One behavior that is often used because it is both ecologically relevant and straightforward to characterize is neophobia, or the fear of novelty. Neophobia has been quantified in several wild bird species by placing a novel object on or near the animal's food dish, and timing the latency to approach relative to an unmodified dish. In developing the neophobia assay as a tool for use in avian stress research in

our lab, I observed that house sparrows caught in October showed very different behavior than animals caught in July. Just as time of year may affect the physiological response to capture (see section 2.9), it may also impact the behavior of neophobia in captivity. In Chapter 6, I present data from neophobia trials run on birds caught at different times of year. *I hypothesized that time of year would influence neophobia, with the birds perhaps being less neophobic during the breeding season, when they are more highly food-motivated.*

The different hormonal pathways involved in the stress response are not completely independent. Just as glucocorticoids can affect the adrenomedullary system, both hormonal systems can impact behavior. Fearfulness of new objects may be tied to the stress response – novelty may trigger a hormonal response that increases fearfulness and drives behavior. Glucocorticoids have inconsistent effects on neophobia in birds. However, the impact of the adrenomedullary system on neophobic behavior has not previously been investigated. In Chapter 7, I present the results of an experiment determining whether elevated heart rate is associated with novelty, thus linking behavior and the adrenomedullary system. In this experiment, I used European starlings (*Sturnus vulgaris*) as my study species. *I hypothesized that exposure to novel objects would cause an increase in heart rate compared to an unmodified food dish.*

CHAPTER 2: CHRONIC CAPTIVITY STRESS IN WILD ANIMALS

Clare Parker Fischer and L. Michael Romero

2.1 Abstract

Wild animals are brought into captivity for many reasons – conservation, research, agriculture, and the exotic pet trade. In captivity, the physical needs of animals are met. However, the conditions of confinement and exposure to humans can result in physiological stress. The stress response consists of the suite of hormonal and physiological reactions to help an animal survive potentially harmful stimuli. The adrenomedullary response results in increased heart rate and muscle tone (among other effects) and elevated glucocorticoid hormones help to direct resources towards immediate survival. While these responses are adaptive, overexposure to stress can cause physiological problems, such as weight loss, changes to the immune system, and decreased reproductive capacity. Many people who work with wild animals in captivity assume that they will eventually adjust to their new circumstances. However, captivity may have long term or permanent impacts on physiology if the stress response is chronically activated. In this review, we analyzed the effects of captivity on the physiological systems impacted by stress, particularly weight changes, glucocorticoid regulation, adrenomedullary regulation, and the immune and reproductive systems. We found that adjustment to captivity has been reported for some physiological systems in some species. However, for many species, permanent alterations to physiology may occur with captivity. For example, many species have elevated glucocorticoids and/or reduced

reproductive capacity compared to free-living animals even after months in captivity. Full adjustment to captivity may occur only in some species, and may be dependent on time of year or other variables. We discuss some of the methods that can be used to reduce chronic captivity stress.

2.2 Introduction

The tens of thousands of vertebrate species on this planet are adapted to every condition from the Arctic to the tropics. For all of these species, the environment contains both predictable changes (e.g. day-night transitions or seasonal variation) and unpredictable, uncontrollable threats to homeostasis and survival (Romero and Wingfield, 2016). Vertebrates have evolved a suite of defenses against the myriad unpredictable "shocks that flesh is heir to" (Shakespeare, *Hamlet*, 3.1)– a set of conserved physiological responses known as the "stress response." However, while the stress response can help survive a threatening event, if noxious conditions are repeating or unrelenting two physiological changes take place. First, the reactive scope of the animal shrinks thereby decreasing the animal's ability to cope (Romero *et al*., 2009). Second, the stress response itself can begin to cause physiological problems, a condition known as "chronic stress." Chronic stress can lead to weight loss, immunosuppression, reproductive failure, and psychological distress (Sapolsky *et al*., 2000). Because the stress response occurs when situations are perceived as threatening, whether or not the animal is experiencing real physical harm, a drastic change of conditions can lead to chronic stress, even when the animal is unharmed. When a wild animal

is brought into captivity for the first time, chronic stress can occur even though the physical needs of the animal are attended to.

In captivity, animals are provided with shelter and ample food. Nevertheless, captivity can often result in negative physiological outcomes, particularly for newly-captured animals. The conditions of captivity can be perceived as threatening, and if the perceived threat does not decrease, chronic stress may result. The sources of stress in captivity are many, including cage restraint, human presence, an unfamiliar environment, and other, more subtle stressors, such as artificial light conditions (reviewed in Morgan and Tromborg, 2007).When wild animals are newly brought into captivity, it is frequently for research, conservation, agriculture (e.g. fisheries), or the exotic animal trade. To keep these animals healthy, chronic stress should be minimized or eliminated. It is often assumed that with time, animals will adjust to captivity conditions and stress will disappear. Indeed, many animals seem to thrive in captivity; unfortunately, many other species do not (Mason, 2010). In this review, we surveyed the literature in order to answer the following two questions: do wild animals eventually adjust to captivity conditions? And if so, how long does the period of adjustment typically take?

We focused on several aspects of physiology that may be particularly affected by chronic stress. The acute stress response involves two hormonal pathways. The adrenomedullary response occurs within seconds of a sudden loud noise (Romero and Wingfield, 2016). The catecholamine hormones epinephrine and norepinephrine are rapidly released from the adrenal medulla. These cause an

increase in heart rate, as well as an increase in muscle tone, an increase in blood pressure, and other physiological and behavioral changes that enable an animal to survive a sudden stressor, such as a predator attack. Within minutes of the onset of a stressor, a hormonal cascade triggers the synthesis and release of glucocorticoids (GCs) – steroid hormones that have wide-ranging effects on the body (Romero and Wingfield, 2016). While baseline levels of GCs help regulate metabolism, increased levels trigger an "emergency life history stage," where resources and behaviors are directed towards survival of the crisis and away from long term projects. The immune system is strongly affected by GCs, as is the reproductive system (Sapolsky *et al*., 2000). In this review, we focus on captivity's effects on mass (one of the best-documented outcomes of chronic stress), GC concentrations, and the immune, reproductive and adrenomedullary systems. We also document how the adjustment to captivity is impacted by time of year and how captivity effects persist after release. Finally, we discuss some of the ways that captivity stress may be mitigated.

2.3 Methods

We surveyed the literature and gathered studies that compared wild-caught animals as they adjusted to captivity. We conducted a literature search through Web of Science using the search terms "captivity" and "stress" and "physiology" or "endocrinology" and related words. Because many papers reported on aspects of the stress response on animals that were in captivity but did not examine the effects of captivity itself, we were unable to devise search terms that included the studies we were interested in but excluded other research on stress in wild animals. We

therefore devised the following criteria to determine whether papers should be included: 1) wild species were brought into captivity and physiological variables measured over the days to months of adjustment to captive conditions OR 2) wild-caught captive animals were compared to free-living conspecifics AND 3) the total captivity duration was at least 3 days (we did not include the many studies that measure only the acute stress effects of capture in the first 30 min to 48 hours). We excluded studies where we could determine that all captive animals were captive-bred, as we were specifically interested in how well wild animals can adjust to captive conditions when taken from the wild (though we included some studies where the origin of captive animals was unclear). Once we had created a list of papers, we also looked through the cited references of these studies to look for any important works our search terms missed.

There are many studies that focused on behavioral changes in captivity. However, the variables measured can be quite species-specific and difficult to interpret in a context of stress. Although we recognize the importance of behavior for the welfare of wild animals (reviewed in McPhee and Carlstead, 2010), we limited our focus to studies that included some physiological measurements (e.g. weight changes, hormone concentrations, or immune measurements).

We found little standardization in experimental design in the papers examining the effect of captivity on physiology. We visually summarize the four most common experimental designs in Figure 2.1. Many researchers compared animals that had been exposed to captivity (duration: 3 days to several years) to those that had not (Figure 2.1A). In some cases, the free-living population was

sampled when the captive population was initially captured. This was often the case in species where only a single blood sample could be drawn from an individual. In other studies, the free-living population was sampled entirely separately from the captive group. This was often the case for long-term captives, such as zoo-housed animals. Another common technique was to take a single pre-captivity sample and a single post-captivity sample on the same animal (duration of captivity 5 days-3 months) (Figure 2.1B). Other researchers used repeated sampling techniques – either sampling the same individual multiple times, or keeping different individuals in captivity for different durations before sampling. Some focused narrowly on the first few days of captivity (Figure 2.1C), while others did not take a second sample until several weeks had passed (Figure 2.1D).

We created summary figures for the trends we observed in weight, glucocorticoid hormones, and the immune system with respect to captivity duration (Figures 1.2, 1.3, and 1.4). To construct these, we tallied the total number of studies that reported on the variable for a particular time window and determined whether the variable was above, below, or equal to what it was in a free-living population. If a single report showed two different patterns (for example, males and females had different patterns or two species were reported in the same paper), each pattern of was included separately. Therefore, one "study" might be included multiple times in the figure. This also holds true for reporting patterns in the literature in the text and in the tables – if one paper reported multiple patterns in different groups of individuals, it was included more than once in calculating percentages of studies and was given more than one line on the tables. We did not include studies in the

figures if there were marked seasonal differences in one species (see section 2.9 for seasonal differences).

2.4 Mass and body condition in captivity

After being brought into captivity from the wild, animals frequently experience a period of weight loss (Table 2.1). In 63% of studies (20 of 32), there was a documented decrease in mass associated with captivity during at least the initial capture period. Weight loss in captivity is likely to be attributable to chronic stress. Captive animals are not calorically restricted (as long as they choose to eat), which is not always the case in the wild, and they are not likely to use as many calories because cage restraint limits the amount of exercise that an animal can get in a day. Experimentally induced chronic stress has been demonstrated to lead to weight loss in mammals (e.g. Flügge, 1996), birds (e.g. Rich and Romero, 2005), and fish (e.g. Peters *et al.*, 1980). In fact, weight loss is the most consistently seen effect of chronic stress (Dickens and Romero, 2013).

In 45% of studies where animals lost weight (9 of 20), the animals eventually regained the weight they had lost. In some cases, weight loss may be very transitory and last only a couple of days. For example, North Island saddlebacks (a bird native to New Zealand) lost weight on the first day of captivity but had not only regained weight, but weighed more than their at-capture mass by day 3 (Adams *et al*., 2011). In these cases, weight loss may be related to adjustment to their captive diet, including primarily water loss, and not to major physiological problems. In other species, it may take weeks or months to recover to pre-captivity weights. House sparrows brought into captivity have well-documented weight loss

at 5-7 days post-capture (Fischer and Romero, 2016; Lattin *et al*., 2012a). In a long-term study of the species, they did not regain the weight they had lost for nearly 5 weeks (see Chapter 3). Similarly, female possums lost weight for 5 weeks before beginning to gain again, and although they were kept for 20 weeks, they never recovered fully to their at-capture weight (Baker *et al.*, 1998). In 55% of studies (11 of 20), weight that was lost was never regained, though the studies may not have been long enough for weight to stabilize.

In some cases, weight loss depended on the characteristics of the animal at capture. For example, while all female possums lost weight over the first 5 weeks of captivity, some males gained weight during that period (Baker *et al.*, 1998). When curve-billed thrashers were captured, birds from urban environments had higher body condition than desert birds, but after 80 days in captivity, their body conditions had converged to an intermediate value, though neither urban nor desert birds were significantly different from starting conditions by the end (Fokidis *et al*., 2011). This may be an indication that the chronic stress of captivity acts differently on different individuals depending on their sex, population of origin, or other individual characteristics. An individual may also respond differently to captivity depending on its own physiological state (see section 2.9 for the effects of time of year on the ability to adjust to captivity).

Weight loss was not the only pattern seen in captivity. In 19% of studies (6 of 32), animals gained mass above their starting condition. In captivity, animals are not calorically restricted and are also limited in their ability to burn energy through exercise. While some animals may benefit from increased calories, *ad libitum*

access to food may cause others to become obese and face the myriad negative consequences of a high body mass or body fat content (West and York 1998). In a study of domesticated budgerigars, birds were given ad libitum food and confined to cages that limited exercise. High body mass at the end of 28 days correlated with a greater degree of DNA damage (Larcombe *et al*., 2015). We visually summarized the patterns of weight loss and regain in Figure 2.2. We graphed the total percent of studies that showed weigh gain, weight loss, or no change in weight at different time points after introduction to captivity. There were no studies that recorded weight gain in the first day. Most weight gain seems to be reported at 15-28 days of captivity (38% of studies showed weight gain in that window). The percent of studies reporting weight loss decreased with increasing captivity duration, reflecting the fact that many studies show eventual regain of lost weight. This suggests that for many species where weight was lost, it would eventually be regained.

It is possible that seasonal fluctuations in weight may interfere with the assumptions that weight gain or loss is due to captivity. Captive ruffs and red knots have strong seasonal weight fluctuations in captivity associated with weight gain for migration and breeding (Piersma *et al*., 2000). However, newly-caught ruffs gained weight quickly in the first 2 weeks and never again dipped below their initial average weight. If semi-naturalistic conditions are maintained in captivity (for example, if the animals are exposed to natural day length or are housed outdoors), then they may continue to experience seasonal weight changes that are not due to overfeeding or to chronic stress.

2.5 Changes in glucocorticoids during the adjustment to captivity

One of the most common variables to measure when assessing the stress of captivity was glucocorticoid (GC) concentrations. Glucocorticoid hormones (primarily cortisol in fish and most mammals; primarily corticosterone in reptiles, birds, amphibians, and rodents) are produced in the adrenal cortex, have multiple roles throughout the body, and can influence many other physiological systems. Acute stressors cause a transitory increase in GCs, which is eventually brought down by negative feedback. Chronic stress frequently results in changes in GC regulation, although the part of the GC response affected (baseline concentrations, stress-induced concentrations, or negative feedback) and the direction of the change are different in different species and circumstances (Dickens and Romero, 2013).

GCs can be assessed in several ways (Sheriff *et al.*, 2011). The most common method is to measure circulating plasma GCs by taking a blood sample. The sampling procedure itself can cause an increase in GCs, so researchers usually try to acquire the first sample as quickly as possible – within three minutes of capture or disturbance is generally considered a good guideline (Romero and Reed, 2005). In many studies, it was not possible for the researchers to meet this standard because of the difficulty of capturing and bleeding the animals. In addition, some papers were written before the 3-minute standard had been established. It is also possible to assess GCs through other means. Fecal or urine samples are frequently collected to measure metabolized GCs. Fecal samples provide an integrated profile of GC secretion over several hours and reflect both baseline GCs and acute stress events (Wasser *et al.*, 2000). Fecal sampling is convenient for many species when rapid capture and blood sampling is impractical. If the first fecal sample is collected

soon after capture, it will not reflect the stress of captivity and may be considered a good free-living reference. Some researchers also used urinary GC metabolites, particularly in amphibian species, where animals could be left alone in a container of water from which excreted steroids were measured.

The initial capture and handling of wild animals is expected to cause an increase in circulating GC levels (an acute stress response). While some researchers investigated captivity-induced changes in the acute stress response itself (e.g. taking a plasma sample after a standardized 30-minute restraint stress at capture and again after a period in captivity), others incorporated the acute response to capture in the same analysis as longer-term captivity effects (e.g. taking a sample at 0, 2, 6, 18, 24, 48, and 72 hours post capture). Because of the variety of different measures used, we focused particularly on the captivity effects on baseline and integrated GCs (Table 2.2). However, we will also discuss the effects of captivity on the acute stress response and negative feedback of GC production (Table 2.3). Some researchers looked for the effects of captivity at different times of year – we do not include those studies in our calculations or in Tables 1.2 and 1.3 (see Section 2.9).

Captivity does not influence GCs in all species. In 19% (10 of 54) studies, there was no recorded difference in GCs during or after the captivity period compared to free-living levels. Thus, in a majority of studies, captivity caused a change in baseline or integrated GCs. In 39% of studies (21 of 54), wild animals had increased GCs at the end of the capture period compared to concentrations in free-living animals. For example: garter snakes had higher baseline plasma GCs

after 4 months of captivity than they had at capture (Sparkman *et al*., 2014), curve-billed thrashers had elevated baseline GCs up to 2.5 months after capture (Fokidis and Deviche, 2011), and Grevy's zebras had higher fecal GC metabolites during 6 weeks of captivity than they had at capture (Franceschini *et al*., 2008). Elevated GCs are traditionally interpreted as indicating that animals are chronically stressed. Experimentally induced chronic stress can often lead to elevated baseline GCs, although this is by no means a universal response (Dickens and Romero, 2013). Adrenal hypertrophy may be an underlying mechanism explaining the long-term elevation of GCs. For example, chronic captivity stress led to increased adrenal mass in African green monkeys (Suleman *et al*., 2004) and mouse lemurs (Perret, 1982). In nine-banded armadillos, 6 months of captivity (but not 3 months) caused adrenal changes similar to those after a harsh winter (Rideout *et al.*, 1985) and in herring gulls 28 days of captivity led to adrenal lesions (Hoffman and Leighton, 1985).

However, many studies that reported elevated GC concentrations at the end of the captivity period may eventually have shown decreased GCs had the study been carried out for longer. For example, house sparrows had elevated baseline GCs after 1-7 days in captivity (Fischer and Romero, 2016; Kuhlman and Martin, 2010; Lattin *et al*., 2012a) that were partially reduced after 1 month (Kuhlman and Martin, 2010). When house sparrows were sampled repeatedly over 6 weeks of captivity, baseline GCs peaked at day 7, but then were dramatically reduced over days 11-42 and approached at-capture concentrations (see Chapter 3). Although GCs may still

have been slightly elevated at the end of the six weeks, they were still on a downward trajectory.

The duration of captivity in the studies we collected was quite variable. Some animals had been in captivity for days or weeks at the time of the final sample, while others had been held captive for months or years. To consolidate the patterns from multiple studies of multiple species and with different sampling times, we graphed the percent of studies with elevated GCs (relative to free-living levels) against captivity duration (Figure 2.3). We expected to see decreasing percentages of studies with elevated GCs as captivity duration increased, following the pattern of increased GCs immediately after capture followed by a decrease towards free-living levels as the animals adjusted to captivity conditions (e.g. Figure 2.1C-D). This is a typical *a priori* prediction in the literature. However, we found that 41% (5 of 12) of species continued to have elevated GCs after 3 months or more of captivity. For example, long-term captive Canada lynx (Fanson *et al.*, 2012), African wild dogs (Van der Weyde *et al.*, 2016), and spider monkeys (Rangel-Negrin *et al.*, 2009) had higher fecal glucocorticoids than free living populations. This suggests that for many species, there is never a complete adjustment to captivity. It is also possible that a publication bias exists in the papers we collected. When researchers did not see a difference between long term captives and free-living animals, they may have been less likely to publish, or perhaps included those results in other studies that did not appear in our literature searches. It is interesting to note that the fewest studies reported elevated GCs at around two weeks post captivity, the amount of time that many researchers allow for their study

species to become acclimated to laboratory conditions (e.g. Davies *et al*., 2013; Lattin and Romero, 2014; McCormick *et al.*, 2015).

The analysis in Figure 2.3 has serious weaknesses in that it contains many different taxa, study designs, durations, etc. A better way to test whether GCs concentrations change over the duration of captivity is to use a repeated-measures design where samples are collected from the same individuals at multiple timepoints post capture. Alternatively, if multiple samples cannot be collected from the same individual, a number of individuals could be collected at once and separated into different groups to be sampled after different captivity durations. However, even in study designs with repeated sampling, only 47% of studies (16 of 34) showed an early increase in GCs followed by a decrease back to free-living levels (e.g. Figure 2.1C-D). Of the remaining studies, 29% (10 of 34) matched the pattern in Figure 2.3 with no decrease in GC concentrations over time, 12% (4 of 34) showed decreased GC concentrations in captivity, and 12% (4 of 34) reported no change in GCs whatsoever. When an early peak was followed by a decrease, this peak and fall sometimes occurred quite quickly. For example, mouse lemurs had increasing fecal GC metabolites over the first day of captivity, but these decreased to at-capture levels by day 5 (Hämäläinen *et al*., 2014). On the other hand, some species required a long adjustment period. For example, the Fijian ground frog had elevated urinary GCs until day 25 post capture (Narayan and Hero, 2011).

In some studies, with repeated measures designs, the researchers did not or could not obtain a sample that represented free-living animals. In these cases, the

first sample could not be acquired for minutes, hours, or even days after capture, and the acute stress of capture and handling was likely reflected in high early GC concentrations. In all 9 studies where this was the case (see Table 2.2), an initial high concentration of GCs then decreased over the study period in at least some animals. This is consistent with the pattern we expected to see in Figure 2.3, where capture and the initial transfer to captivity results in high GCs that then decrease as the animal adjusts to capture. For example, female brushtail possums were not sampled until days after their capture and transfer to captivity, but showed decreasing plasma GCs from week 1 to week 20 of captivity (Baker *et al.*, 1998). This pattern was not seen in males, however, and without a free-living baseline, it is impossible to determine whether males could not adjust to captivity, or whether they did so within the first week, though the males did have substantially lower GCs than females at the first sample (Baker *et al.*, 1998).

These studies on baseline GCs together demonstrate a pattern wherein approximately half of species appear to adjust to captivity as evidenced by the elevated GC levels becoming reduced the longer they are exposed to captive conditions. Although some species seem to take longer to acclimate to captive conditions than others, it appears that many species will eventually show a reduction in GCs after an initial peak. We see this pattern across taxonomic groups, in birds, fish, reptiles, amphibians, and mammals. However, we should be careful to not to interpret a reduction in circulating baseline GCs, fecal GC metabolites, or urinary GCs as a complete adjustment to captivity and an elimination of chronic stress. Even when baseline GCs have returned to free-living levels, other aspects of

the animals' physiologies may be negatively impacted. For example, even though circulating GCs were only elevated for one day in African green monkeys, adrenal mass was almost doubled after 45 days in captivity (Suleman *et al.*, 2004). Similarly, while it is tempting to conclude that elevated GCs are diagnostic of chronic stress, it should be kept in mind that baseline GCs have many functions in metabolism and energy use. A change of baseline GCs in captivity could merely reflect a change in energy requirements and not the physiological damage we associate with chronic stress.

Furthermore, in 13% (7 of 54) of studies, captivity induces a temporary or permanent reduction in baseline GC levels. For example, chukar partridges had reduced baseline and stress induced GCs after 5 and 9 days of captivity (Dickens *et al.*, 2009b) and black rhinoceroses had reduced fecal glucocorticoid metabolites over 60 days of captivity (Linklater *et al*., 2010). This could be interpreted as a reduction in allostatic load on these animals, and the chukar indeed gained weight over this period (Dickens *et al.*, 2009b). However, the rhinoceroses had severely reduced plasma sex steroids during captivity, indicating that they may indeed be negatively physiologically impacted (Linklater *et al.*, 2010). A reduction in GCs could be interpreted as exhaustion of adrenal capacity, and could also be a sign of chronic stress (Dickens and Romero, 2013).

2.5.1 Impact of captivity on acute stress response and negative feedback of GC production

Relatively few researchers have explicitly investigated the effects of captivity on the acute GC stress response (see Table 2.3). Of those that have, 62% (10 of 16) found no effect of captivity (captivity duration 5-80 days). The six studies

that reported changes in stress-induced GCs showed changes in opposite directions. In two studies, stress-induced GCs were decreased in captivity, while in 4 studies, stress-induced GCs were increased in captivity.

The negative feedback of the GC response to stress, where high GC levels lead to the inhibition of GC production, is very important for the control of physiological stress. Although chronic stress has frequently been found to affect the negative feedback of GC production (Dickens and Romero, 2013), we found only two studies that explicitly measured negative feedback strength in animals immediately at capture and after a period of captivity. In both cases, animals were injected with a synthetic GC (dexamethasone) after mounting a stress response to stimulate maximum negative feedback. The strength of negative feedback increased slightly in house sparrows after 5 days of captivity (Lattin *et al*., 2012a), whereas negative feedback strength decreased after 5 days of captivity in chukar partridges but returned to its at-capture strength by 9 days (Dickens *et al.*, 2009b). This is an important aspect of stress physiology, one that is critical for the total amount of GC exposure, and warrants further study to determine whether it is impacted by the stress of captivity in many species.

2.6 Immune consequences of captivity

Stress has well-documented, but sometimes complex, effects on the immune system. In large part, these changes are due to the acute or long-term effects of elevated GCs on leukocyte populations. GCs can cause immune redistribution, moving lymphocytes out of the bloodstream and into the skin, spleen, and lymph nodes, where they will be available in case of a wound (Dhabhar

and Mcewen, 1997; Johnstone *et al.*, 2012). GCs can also cause proliferation or mobilization of neutrophils (most vertebrates) or heterophils (birds and some reptiles) (Dale *et al.*, 1975; Gross and Siegel, 1983; Johnstone *et al.*, 2012). Together, these effects on leukocyte populations result in a change in the neutrophil or heterophil to lymphocyte ratio (N or H:L ratio) (Dhabhar and Mcewen, 1997; Johnstone *et al.*, 2012). A change in the N or H:L ratio does not necessarily mean that an animal's immune system is hypo- or hyperactive. Instead, this acts as another metric for GC secretion. A long-term increase in N or H:L ratio, like a long-term increase in circulating GCs, can be an indication that an animal is suffering from chronic stress.

We summarized the 23 studies that reported leukocyte counts in Table 2.4. Although the N or H:L is a useful metric, in some studies the researchers chose to report total number or percent of different leukocyte types without calculating or performing statistics on the relative abundances of neutrophils/heterophils and lymphocytes. In these cases, we inferred the direction (or presence) of change after captivity of the N or H:L ratio based on the changes in leukocyte counts or percentages that were reported. In 2 studies, only the total number of leukocytes was reported without further subdivision of leukocyte types. In 48% of studies (10 of 21), N or H:L ratio was elevated at the end of the measured captivity duration relative to its free-living value. 29% of studies (6 of 21) documented no change in N or H:L ratio over the study period. N or H:L ratio was decreased in 24% of studies (5 of 21). In one of the studies that showed a decrease of H:L ratio, the first sample was taken after 1-2 days in captivity and a minor surgical procedure, so the true

free-living measure was unrecorded (Kuhlman and Martin, 2010). In one study (in the Fijian ground frog), the N:L ratio was elevated for 15 days in captivity, but then returned to wild levels by day 25 (Narayan and Hero, 2011), resulting in no overall change. We summarized the overall patterns of N or H:L ratio compared to captivity duration in Figure 2.4. The number of studies reporting an increase in N or H:L ratio decreases with captivity duration. This suggests that many or most species do adjust to captivity, and an initially high N or H:L ratio may decrease given sufficient time.

A change in the N or H:L ratio is generally interpreted to represent a change in distribution of leukocytes, not necessarily a change in leukocyte numbers. Lymphocytes, in particular, may decrease in circulation with high GC concentrations. Fewer lymphocytes in circulation could mean that they have been destroyed and the animal therefore would be expected to have reduced adaptive immune capacity. However, it could also represent a change in the location of different leukocyte types within the body. Lymphocytes that are not circulating might still be functioning properly – they have simply moved to where they might be needed, infiltrating the skin, lungs, or intestines where they will be on-site in case of exposure to infectious agents. Kuhlman and colleagues investigated the redistribution of leukocytes to the skin (Kuhlman and Martin, 2010). They implanted a gelatin sponge under the skin of house sparrows on day 0, day 1-2, or after 1 month of captivity. Leukocytes were allowed to infiltrate into the sponge for 24 hours. Then the sponge was explanted and leukocytes were counted in circulation and in the sponge. They found that lymphocytes decreased in both the

blood and the sponge after 1-2 days in captivity and remained depressed for 1 month. Heterophils were unchanged in circulation, but in the sponge, there were fewer heterophils after 1-2 days of captivity but more after 1 month compared to wild birds. They concluded that these data did not provide evidence for immune redistribution as the total numbers of leukocytes in the sponge and circulation were not correlated.

Some studies also reported the total leukocyte counts, sometimes without further subdividing them into classes. While decreased circulating leukocytes has been associated with stress (generally because of redistribution rather than destruction of cells) (reviewed in Dhabhar, 2002), there was no clear pattern with the number of leukocytes in captivity. 53% of studies (9 of 17) showed no change in total white blood cells compared to free-living animals by the end of the captivity period; 23.5% (4 of 17) showed a decrease in circulating leukocytes; and 23.5% (4 of 17) showed an increase (captivity duration 3 days to 1 year, see Table 2.4).

Neither total leukocyte numbers nor the N or H:L ratio provide a very strong indication of immune capacity, however. In order to more directly assess immune functionality in captivity, some researchers have used other, more functional, measurements. The bacterial killing assay is a way to determine how effectively fresh whole blood is able to eliminate bacteria. This assay has the advantage of determining the real effectiveness of the immune system against pathogens (Millet *et al*., 2007). In the cururu toad, whole blood was less effective at killing bacteria after 13 days of captivity (de Assis *et al*., 2015). Similarly, in red knots held in captivity for 1 year, whole blood was less effective at eliminating two

Staphylococcus species than in wild living birds (though there was no difference in *E. coli* elimination) (Buehler *et al.*, 2008).

Another way to measure immune responsiveness is by measuring a proliferative response against non-specific antigens. In some studies, this is done by culturing a sample of blood along with an antigen and quantifying cell division. In brushtail possums, proliferation of leukocytes in response to the non-specific plant toxin phytohemagglutinin was measured (Baker *et al.*, 1998). Male brushtail possums showed a decrease in proliferative response over 20 weeks, which increased again in 1 year. In female possums, the proliferative response increased from 11 to 15 weeks in captivity, and then remained at that high level for at least a year (Baker *et al.*, 1998). In another study in male brushtail possums, leukocyte proliferation to a *Mycobacterium* protein derivative increased after 4 and 6 weeks of captivity, but only when the animals were housed in high-density pens (Begg *et al.*, 2004). The proliferative response to phytohemagglutinin can also be measured in-vivo if PHA is injected into the skin and the degree of swelling is quantified. In zebra finches, there was no difference in the in-vivo PHA response between newly captured birds and those held for 10 or 16 days (Ewenson *et al.*, 2001).

Two studies have attempted to quantify the strength of the adaptive immune system in captivity. In red knots, plasma was plated with rabbit red blood cells. The degree of hemolysis and hemagglutination provided a measure of complement and natural antibody action. Hemolysis and hemagglutination were similar in wild and captive birds when they were measured at the same time of year, which indicates that the strength of the adaptive immune response is unaffected by captivity

(Buehler *et al., 20*08). Newly captured killifish had stronger response to antigen after immunization than 4-6 week captives, indicating that the adaptive immune system was less effective after captivity (Miller and Tripp, 1982).

Overall, there does not seem to be a single pattern for immune regulation with captivity. While captivity has been shown to repress immune function in some species (e.g. reduced bacterial killing in red knots and cururu toads), in other species, the immune system may be hyperactivated. For example, in house sparrows, gene expression for pro-inflammatory cytokines was elevated in captive birds (2 and 4 week captives) compared to newly caught animals, which was interpreted as hyperinflammation in captive birds (Martin *et al*., 2011). Changes in the immune response with chronic stress are thought to be most strongly tied to GC release. However, the impacts of GCs on the immune system can be complex. In the short term, GCs typically induce an immune response, while they can be immunosuppressive over the long term, although these interactions tend to be context-dependent (Dhabhar and Mcewen, 1997; Martin, 2009). As the interaction between GCs and immunity is complex and context specific, and as the interaction of GCs to captivity can be complex as well, it is not currently possible to predict whether captivity conditions result in appropriate or inappropriate immune activity.

2.7 Effects of captivity on the reproductive system

Captivity has well-documented negative impacts on reproductive biology. In many species, captive breeding for research or conservation purposes can be a challenge. For example, cheetahs and pandas are notoriously difficult to breed in captivity. Even the house sparrow so commonly used as a model species does not

readily breed in captivity (Lombardo and Thorpe, 2009). In 71% of studies (15 of 21), the transition to captivity resulted in reduced reproductive capacity in wild species (Table 2.5). Note, however, that these papers do not cover an extensive literature on captive breeding, including in individuals who have spent decades in captivity or were born in captivity, which is beyond the scope of this review. Here, we focus only on those papers that studied reproductive capacity of recent captives (only within the first year) and that examined a mechanism for reduced reproduction (i.e. the study didn't only report a failure to breed). There was no obvious taxonomic pattern for species that had reduced reproductive ability in captivity vs those that had no documented reproductive problems. Duration of captivity did not appear to be a factor either. In one study of water frogs, reproduction in both males and females were negatively impacted by only 3 days of captivity (Zerani *et al*., 1991), while in jack mackerel, reproduction was inhibited after a full year of time to adjust to captivity (Imanaga *et al*., 2014).

Different researchers measured different variables for reproductive capacity. Many studies analyzed reproductive steroid hormones (primarily testosterone in males and estrogen and/or progesterone in females). However, other variables were also measured, including gonad size and development, behavior, and gamete development. In house sparrows, Lombardo and Thorpe found decreased sperm production, reduced testes size, and a change in beak color from breeding-season black to wintering brown after 3 months of captivity (Lombardo and Thorpe, 2009). Female anole lizards experienced a rapid decrease in plasma vitellogenin (a protein that is necessary for yolk production), which was followed by regression of

developing follicles (Morales and Sanchez, 1996). In electric fish, behavioral differences between males and females were reduced in captivity until they disappeared or even reversed. This occurred concurrently with decreases in testosterone and 11-ketotestosterone (a potent androgen in fish) in males (Landsman, 1993).

The reduction of reproductive capacity might be tied to GC levels. GCs can be powerful suppressors of reproductive steroids (Sapolsky *et al.*, 2000). Prolonged GC exposure can lead to decreased production of testosterone or estradiol, which can then have downstream effects on gonad development, egg maturation, sperm production, and behavior. In green treefrogs, a decrease in sex steroids was concurrent with an increase in GCs (Zerani *et al*., 1991). However, in black rhinos, males had suppressed fecal testosterone and females had suppressed fecal progestins even though GC levels were below free-living levels for most of the captivity period (Linklater *et al*., 2010).

Captivity did not always show the expected suppression of reproduction. In most studies that did not show an effect of captivity, reproductive hormones were the only variables measured and they showed no change. The only exception was in the brown treesnake, where 3 days of captivity did not affect testosterone, but also did not affect ovarian development (both were very low in free-living and captive animals) (Mathies *et al.*, 2001). However, another study in brown treesnakes found underdeveloped testes in males after 4-8 weeks of captivity, even though they were the same body size of mature males in the wild (Aldridge and Arackal, 2005). Captivity may affect sexual variables differently in males and

females. For example, in water frogs held in captivity for 2 weeks, only males appeared to be negatively affected by captivity (Gobbetti and Zerani, 1996).

Overall, it appears that captivity tends to have a negative impact on reproduction in most species. However, there are relatively few studies that specifically examine the reproductive physiology of newly-captured animals. Furthermore, given that many animals eventually do breed in captivity while others do not, it is not clear how long-lasting these impacts may be or why they impact some species more than others. This is an area that needs more study.

2.8 Adrenomedullary effects of captivity

The adrenomedullary arm of the stress response can be difficult to measure. Measuring epinephrine or norepinephrine in the blood is relatively straightforward, but these hormones increase within seconds of disturbance, meaning that acquiring a free-living baseline in a wild animal is virtually impossible. We excluded most studies that measured epinephrine or norepinephrine, as sampling techniques between wild and captive animals sometimes differed in ways that would obscure the meaning of their results. Recording heart rate is another way to infer activity of the adrenomedullary system (Romero and Wingfield, 2016). Heart rate recordings typically involve the use of specialized and expensive equipment, but can give instantaneous updates on heart rate. In addition, scientists can measure heart rate variability, which gives a metric of how much relative control the sympathetic and parasympathetic nervous systems have over heart rate (Romero and Wingfield, 2016). However, depending on the type of heart rate recording device, it may be impossible to obtain baseline free-living heart rates. Although some researchers

have had success measuring heart rate in free-living animals (e.g. white-eyed vireos (Bisson *et al*., 2009)), to our knowledge, there has not yet been a study that directly compares heart rate in free-living and captive animals of the same species. Because such limited data exist on the adrenomedullary effects of captivity, we also included captive-raised animals in our literature search.

Some researchers have directly measured plasma epinephrine or norepinephrine in captive and free-living animals. Plasma norephineprhine under anesthesia (collected within 50 minutes) decreased over 19 months of captivity in rhesus macaques, though a free-living sample could not be obtained under the same conditions (Lilly *et al.*, 1999). Captive-raised bighorn sheep had a higher epinephrine response to a drop-net capture technique than did free-living sheep, though they had similar norepinephrine responses (Coburn *et al*., 2010).

Heart rate has been measured in only a few species during the transition to captivity. In bighorn sheep newly brought into captivity, heart rate during restraint and blood sampling decreased from days 1-2 (when the animals were handled extensively and transported) until day 14 (Franzman and Thorne, 1970). When heart rate from newly-captured European starlings was compared to birds that had been held for more than a year in captivity, heart rate was high at capture but decreased to the level of long-term captives within 24 hours. Heart rate variability was low shortly after capture (indicating increased sympathetic nervous system drive), but increased in 48 hours to long-term captive levels (Dickens and Romero, 2009). However, there was a more profound effect on the heart rate response to a sudden noise. While long term captives showed a robust increase in heart rate after

a loud noise, a typical adrenomedullary response, newly-captured birds had a virtually eliminated heart rate response for at least 10 days. In contrast, the adrenomedullary response to captivity was slightly different in house sparrows. Daytime heart rate was elevated above 1 month captive levels for at least 7 days post-capture (Fischer and Romero, 2016; and see Chapter 5). Heart rate variability tended to increase over the first week of captivity. However, there was no difference between newly-captured and 1 month captives in heart rate variability. These data led to a long-term repeated-measures investigation during the first 6 weeks of captivity (see Chapter 3). Heart rate tended to decrease until day 18, then plateaued. Heart rate variability tended to decrease slightly overall, though there may have been a slight tendency to increase over the first week and then decrease.

Overall, it appears that captivity can cause increased heart rate in wild animals, potentially for weeks or months. Prolonged elevated heart rate has been associated with mortality in captive animals (Domingo *et al*., 1991; Perret, 1982). Moreover, a reduction in the startle response (as demonstrated in European starlings) could have negative consequences for animals that are released from captivity into the wild. The adrenomedullary response to sudden noises or other startling events is an adaptation that allows animals to survive sudden traumatic events, such as predator attacks or conspecific aggression. An impaired startle response could result in death if it persists after the animals are released from captivity. However, there are few studies examining the effects of captivity on the adrenomedullary response. The patterns we see in European starlings and house sparrows are different – it does not appear that there is a consistent heart rate

response to captivity in passerine birds, much less in all vertebrates. We believe this is an area ripe for future studies. As telemetry equipment becomes cheaper and more available, we hope to see more investigations into the adrenomedullary response to captivity and other stressors.

2.9 Effects of captivity on seasonality of hormone regulation

A number of studies examined seasonal differences in the response to captivity. Table 2.6 shows that the time of year when animals are introduced to captivity can have a profound effect on hormonal changes. For example, baseline GCs might increase when free-living birds are in molt, decrease when free-living birds are breeding, and not change when free-living birds are captured during the winter or spring (Romero *et al*., 1999). Furthermore, Table 2.6 indicates that there is no consistent pattern across seasons or taxonomic groups. The implications of these differences are currently unknown, but the season of capture might partly explain the large variation across studies summarized in Figures 1.2-1.4. Understanding why there are seasonal differences in the acclimation to captivity would be an important contribution to this field.

2.10 Other physiological consequences of captivity

Some studies, primarily in marine mammals, reported the effects of captivity on thyroid hormone. Long term captive harbor porpoises had the same thyroid hormone levels as wild populations (Siebert *et al*., 2011). In one study of beluga whales, thyroid hormone decreased over the first few days of captivity, but increased to a long-term stable level by day 11 (Orlov *et al*., 1991). However, in another study in the same species, thyroid hormone decreased within the first few

days and remained low throughout 10 weeks of captivity (St Aubin and Geraci, 1988). Similarly, rehabilitated harbor seal juveniles held in captivity for 4 months had lower thyroid hormone than free-living juveniles (Trumble *et al*., 2013). In female brushtail possums, thyroid hormone was elevated from weeks 6-13, the same period when the animals were regaining weight they had lost in captivity (Baker *et al.*, 1998).

We came across a number of papers that examined other physiological effects of captivity. Some studies reported anatomical changes that may occur in captivity. Mountain chickadees showed remarkable reduction in hippocampal volume after 4 months of captivity (LaDage *et al*., 2009). Black-capped chickadees also had reduced hippocampal size after 4-6 weeks in captivity (Tarr *et al*., 2009). In neither species was the telencephalon effected – the effect was localized to the part of the brain involved in location-based memory tasks. This effect persisted even when the environment was enriched to include memory tasks (LaDage *et al*., 2009). Captivity can lead to various pathologies. In a histological study of mouse lemurs that died spontaneously in captivity, lesions in the kidney were strongly correlated with captivity duration and with adrenal size (Perret, 1982). The investigator also concluded that cardiac disease may result from chronic adrenomedullary stimulation, although they did not measure hormone concentrations directly (Perret, 1982). Herring gulls developed amyloid deposits in the blood vessels of their spleens after 28 days in captivity (Hoffman and Leighton, 1985).

2.11 The persistence of captivity effects after release

The physiological changes caused by captivity can persist even after animals have been released back into the wild. Chukar partridges that were held in captivity 10 days and then released to a new location than where they had originated had lasting changes to their GC regulation (decreased negative feedback for at least 30 days, [Dickens *et al.*, 2009a]). Red foxes that were kept in captivity for 2 to 8 weeks were less likely to establish a stable territory upon release than foxes that were caught and immediately released (Tolhurst *et al*., 2016). River otters kept in captivity for 10 months had lower survival than otters not kept in captivity (Ben-David *et al.*, 2002). The captivity effect was strong enough that crude oil ingestion (mimicking the state of oiled otters in rehabilitation) had no further effect on survival (Ben-David *et al.*, 2002). Rehabilitated barn owls (Fajardo *et al.*, 2000) and guillemots (Wernham *et al.*, 1997) had much shorter life expectancies than wild birds.

However, captivity may not necessarily have lasting negative impacts. In Grevy's zebra, fecal GC metabolites were elevated in captivity, but decreased back to the wild norm quickly after release (Franceschini *et al.*, 2008). Hermann's tortoises kept in captivity for 2-8 years following an injury showed no difference in movement, thermoregulation, or body condition compared to free-living animals after release to the wild (Lepeigneul *et a*l., 2014). Captivity up to 3 months did not affect survival in Stellar's sea lions (Shuert *et al.*, 2015). Captivity may even have positive effects in some cases. For example, hedgehogs were more likely to survive a translocation event if they were held in captivity for greater than 1 month compared to those held less than 6 days (Molony *et al*., 2006).

Whether an animal will be permanently negatively impacted by captivity or not may depend on the captivity conditions, species, time of year, method of release, or individual effects. Wild rabbits held for 2, 4, 6, or 8 weeks in quarantine before release did not differ in survival probability (Calvete *et al*., 2005). In another study in that species, GCs did not change over the course of a quarantine period, but animals with higher plasma and fecal GCs were more likely to survive, even though they had worse body condition (Cabezas *et al*., 2007). Saddlebacks were more likely to survive post-release when they had a robust GC response to a standardized acute stressor (Adams *et al*., 2010). Therefore, captivity may have more profound effects on survival if it negatively and permanently changes GC regulation.

2.12 Amelioration of captivity stress

Captivity can cause a wide number of physiological changes in wild animals that are consistent with chronic stress and are likely to be detrimental to health. However, can anything be done to prevent these changes? Is there a way to protect animals from the negative consequences of captivity stress? While this is not an exhaustive review of the solutions that have been tried, we offer some ideas that have been attempted to relieve chronic stress due to captivity conditions.

Adjusting the physical conditions of captivity may be one of the simplest ways to reduce chronic stress. Transferal from outdoors cages to indoors cages led to reduced reproductive hormones and behaviors in long term captive European starlings (Dickens and Bentley, 2014) and to weight loss and reduced immune function in water voles (Moorhouse *et al*., 2007). Cage size and density are also

important for the development of chronic stress. High density housing during the initial captivity period resulted in elevated GCs compared to low density housing in flounders (Nestor Bolasina, 2011) and wedge sole (Herrera *et al*., 2016). Together, these suggest that housing animals in larger cages at lower density and using outdoors facilities when possible are likely to reduce chronic captivity stress. However, reducing density by caging animals individually can have negative consequences, particularly in social species. Housing brushtail possums in groups eliminated the infection, weight loss, and mortality that were seen when the animals were caged individually (McLeod *et al*., 1997). In male brown headed cowbirds, adding a female to the cage (previously solo housed) resulted in reduced plasma GCs, as well as increased testicular regrowth in photostimulated males, though testes never reached the size of wild males (Dufty and Wingfield, 1986).

Many animals benefit from the use of behavioral enrichments to reduce abnormal behaviors that develop in captivity (reviewed in Mason *et al*., 2007). Enrichments have become standard practice in zoo environments and situations where animals are held long-term or bred in captivity. Enrichments consist of providing animals with the means and motivation to practice a full range of natural behaviors, such as foraging opportunities, exercise opportunities, and places to bathe or dust bathe. Even in temporary or laboratory conditions, environmental enrichments could be relatively easy to supply. However, we were unable to find any papers where the physiological benefits of enrichment techniques were specifically tested in newly captured animals. Trying these techniques as a way to

accelerate the adjustment to captivity in these animals would be an exciting avenue for future research.

Lighting conditions may be very important for visual species. European starlings show more behavioral signs of chronic stress under fluorescent lights with a low flicker rate than a high flicker rate (Evans *et al*., 2012), but the low flicker rate does not elicit a GC response (Greenwood *et al*., 2004). Ultraviolet-deficient lighting resulted in higher baseline GCs in European starlings, although immediately after capture, this stressor may be too subtle to make a difference compared with the other stressors of captivity (Maddocks *et al.*, 2002). Temperature conditions should also be carefully considered, particularly for poikilotherms. Warm conditions during the initial transfer to captivity resulted in high mortality in sardines (Marcalo *et al*., 2008) and higher GCs in cane toads (Narayan *et al.*, 2012).

Overall, by matching captivity conditions as closely as possible to conditions in the wild, with roomy cages, exposure to naturalistic lighting and temperature conditions, and animal densities kept relatively low, many animals will be better able to adjust to captivity and may have reduced chronic stress as a result. However, naturalistic housing conditions may be impractical for many situations. Furthermore, some stressors associated with captivity may be unavoidable. For example, nearly any visual or auditory contact with handlers resulted in a heart rate increase in two red-shouldered hawks (Patton *et al.*, 1985). Therefore, in some cases, it might be beneficial to use pharmaceuticals to reduce chronic stress.

Tranquilizers or sedatives are perhaps the most obvious drug classes to consider using in newly-capture animals. However, these may not actually be particularly effective at eliminating chronic stress symptoms. A long-acting neuroleptic did not result in many physiological changes in newly-caught otters (Fernandez-Moran *et al*., 2004). Regular treatment with tranquilizers did not impact any physiological variable in newly caught impala (Knox *et al.*, 1990). Long-acting tranquilizers reduced behavioral agitation to human approach and handling in red-necked wallabies but did not affect any physiological variable measured (Holz and Barnett, 1996). Similarly, a long-acting tranquilizer changed behavior but not heart rate response to human approach in captive wildebeest (Laubscher *et al*., 2016). The anxiolytic and sedative diazepam did not affect GCs, heart rate, heart rate variability, or activity in house sparrows during the first week of captivity (see Chapter 5). Overall, tranquilizers and sedatives do not appear to have long-term physiological benefits in captive animals. However, they may be useful in the short term. For example, by reducing physical agitation, they may prevent animals from injuring themselves during transport (e.g. in nurse sharks being moved into captivity [Smith, 1992]) or during necessary handling by humans (e.g. in red-necked wallabies [Holz and Barnett, 1996]).

Another strategy for pharmaceutical reduction of chronic stress may be to chemically block the hormones of the stress response. The chemical agent mitotane causes a reversible chemical adrenalectomy, which drastically reduces circulating GCs (Sanderson, 2006). In house sparrows treated with mitotane immediately upon capture, baseline and stress induced GCs were drastically reduced during the initial

captivity period, but recovered to the level of untreated birds by day 10 of captivity (Breuner *et al*., 2000). We investigated the effects of mitotane treatment during the first 7 days of captivity in house sparrows and found that it reduced resting heart rate even when it did not cause the expected dramatic decrease in GC levels (see Chapter 5). The adrenomedullary response can also be pharmaceutically reduced by blocking the receptors of epinephrine and norepinephrine. We used alpha- and beta-blockers (which interfere with binding of epinephrine and norepinephrine to their receptors) during the first week to block chronic captivity stress in house sparrows. We found that while the beta-blocker propranolol had no effect on heart rate, it did prevent the increase in baseline GCs that we typically see in newly-captured members of this species (Fischer and Romero, 2016).

The use of tranquilizers should be deployed to reduce the physical harm an animal may do when it panics due to human presence, transport to another area, or other particularly dangerous events but does not appear to change the physiological effects of captivity stress. Blocking the stress response at a hormonal level may be more effective in reducing the symptoms of chronic stress, though not every treatment is equally effective and the drugs may have other side effects.

2.13 Conclusions

Captivity can cause chronic stress that leads to weight loss, persistent changes in baseline and integrated GCs, changes in N or H:L ratio and other aspects of the immune system, and reproductive suppression. These effects can last for months or years in some species, indicating that some species can never truly adjust to captivity conditions. The welfare implications of chronic captivity stress are

obvious, and zoos and other institutions that hold animals in captivity long-term generally have strategies in place to minimize captivity stress. Breeding facilities (for conservation, research, and agriculture/fisheries) are particularly invested in reducing chronic captivity stress, given its profound impact on the reproductive system. Different aspects of physiology may be more or less affected by chronic captivity stress in the long term. Figure 2.3 indicates that many species may continue to have elevated GCs months or years after capture, while Figures 1.2 and 1.4 suggest that most animals will recover from the weight loss and elevated N or H:L ratios caused by the initial transfer to captivity. Given that weight loss and changes to N or H:L ratio are affected by GCs, it is possible that with continuing high GC concentrations, sensitivity to these hormones decreases in captive animals. The reproductive system tends to be negatively impacted by captivity, presumably because of elevated GC hormones. The negative effects of captivity are be species-specific, some species adjust to captivity while others do not (see also Mason, 2010).

A captive animal may be physiologically quite different than a wild animal (Calisi and Bentley, 2009). Therefore, the confounding effects of captivity must be considered in physiological studies using captive wild animals, even when stress is not the focus of research. Animals that are held in captivity for research might respond quite differently to a range of experimental treatments than a wild, free-living individual would. For example, environmental contaminants had different effects on wild and captive sea otters (*Enhydra lutris*) (Levin *et al*., 2007), and

experimentally induced chronic stress caused a change in fecal GCs in free-living but not captive European starlings (Cyr and Romero, 2008).

The existing literature indicates that the effects of captivity on physiology are inconsistent. Some of the differences between animals that adjust and do not adjust to captivity might be explained by life-history features of the species themselves (see Mason, 2010). For example, captive predators that have large ranges in nature tend to show more behavioral anomalies and more infant mortality than those that naturally have smaller ranges (Clubb and Mason, 2003). However, this area of study may be confounded by seasonal effects. If animals are more susceptible to chronic captivity stress at different times of year, and the vulnerable periods are species-specific, as they appear to be (Table 2.6), then the variation in adjustment time to captivity may be largely explained by seasonal differences. Minimizing captivity stress may be largely a matter of capturing animals at the time of year when they are least vulnerable and managing stress differently throughout the year. Further research into new techniques to accelerate the adjustment to captivity would help researchers and conservationists. Whether it can be reduced or not, captivity stress will continue to be a factor in captive animal research, and the conditions and timing of captivity should be considered as experiments are designed and interpreted.

Table 2.1: Mass changes with captivity in wild animals

Changes in mass during adjustment to captivity	Species		Study design	Timeframe	Citation
Weight gain in captivity	mammals	Steller sea lions (*Eumetopias jubatus*)	repeated measures; pre- vs post-captivity	average 2 months	(Mellish *et al*., 2006)*
		Richardson's ground squirrel (*Urocitellus richardsonii*)	repeated measures: multiple timepoints	14 days	(Hare *et al*., 2014)*
		Columbian ground squirrel (*Spermophilus columbianus*)	repeated measures: multiple timepoints	13 days	(Bosson *et al*. 2009)
		brushtail possums (*Trichosurus volpecula*)	repeated measures: multiple timepoints	37 days	(Day and O'Connor, 2000)*
	birds	ruff (*Philomachus pugnax*)	repeated measures: multiple timepoints	up to 1 year (mass increase in first few weeks, then seasonal fluctuations)	(Piersma *et al*., 2000)*
		North Island saddlebacks (*Philesturnus rufusater*)	repeated measures: multiple timepoints	3 days	(Adams *et al*., 2010)*
No change in mass with captivity	mammals	vervet monkeys (*Chlorocebus aethiops*)[1]	repeated measures: multiple timepoints	8 months	(Kagira *et al*., 2007)
		brushtail possums (*Trichosurus volpecula*) (♂ only)	repeated measures: multiple timepoints	20 weeks	(Baker *et al.*, 1998)*
		brushtail possums (*Trichosurus volpecula*) (♂)	repeated measures: multiple timepoints	8 weeks	(Begg *et al.*, 2004)*
	birds	curve-billed thrasher (*Toxostoma curverostre*)	repeated measures: multiple timepoints	80 days	(Fokidis and Deviche, 2011)*
	reptiles	Duvaucel's gecoks (*Hoplodactylus duvaucelli*)	captive vs. free-living population	>1 year	(Barry *et al.*, 2010)*
	amphibians	cururu toad (Rhinella icterica)	repeated measures; pre- vs post-captivity	13 days	(de Assis *et al*., 2015)*
Weight loss in captivity	mammals	beluga whale (*Delphinapterus leucas*)	repeated measures; pre- vs post-captivity	10 weeks	(St Aubin and Geraci, 1988)*
		harbor seal (*Phoca vitulina*) (juveniles)	captive vs. free-living populations[2]	>4 weeks	(Trumble *et al*., 2013)*

Changes in mass during adjustment to captivity	Species		Study design	Timeframe	Citation
		African green monkey (*Cercopithecus aethiops*)	multiple timepoints; different individuals	45 days	(Suleman *et al*., 2004)*
		bighorn sheep (*Ovis canadensis canadensis*)	repeated measures: multiple timepoints	14 days	(Franzman and Thorne, 1970)*
	birds	zebra finches (*Taeniopygia guttata*)	captive vs. free-living population	60 days[3]	(Ewenson *et al*., 2001)*
		rufous-collared sparrows (Zonotrichia capensis)	captive vs. free-living population	2 weeks	(Ruiz *et al*., 2002)*
		great tit (Parus major)	repeated measures; pre- vs post-captivity	1 week	(Krams *et al*., 2013)*
		house sparrow (*Passer domesticus*)	repeated measures; pre- vs post-captivity	1 week	(Fischer and Romero, 2016)*
		house sparrow (*Passer domesticus*)	repeated measures; pre- vs post-captivity	1 week	Chapter 5*
		house sparrow (*Passer domesticus*)	repeated measures; pre- vs post-captivity	5 days	(Lattin *et al*., 2012a)*
	fish	electric fish (*Gnathonemus petersii*)	repeated measures; multiple timepoints	37 days	(Landsman, 1993)*
Weight lost then regained in captivity	mammals	rhesus macaques (Macaca mulatta)	repeated measures; multiple timepoints	Weight decreased by week 5, increased through 1 year	(Lilly *et al*., 1999)*
		European wild rabbits (*Oryctolagus cuniculus*)	repeated measures; pre- vs post-captivity (different durations)	Weight decreased by week 2, increased and stabilized by week 4[4]	(Calvete *et al*., 2005)*
		brushtail possums (*Trichosurus volpecula*) (♀ only)	repeated measures: multiple timepoints	Weight decreased over 5 weeks, increased through week 20	(Baker *et al*., 1998)*
		tuco-tuco (*Ctenomys talarum*)	repeated measures: multiple timepoints	Weight loss on days 10 and 20, regained by day 30	(Vera *et al*., 2011)*
	birds	white-crowned sparrow (*Zonotrichia leucophrys*)	repeated measures: multiple timepoints	Weight loss at day 1, increased through day 14	(Wingfield *et al*., 1982)*

Changes in mass during adjustment to captivity	Species		Study design	Timeframe	Citation
		greenfinch (*Chloris chloris*)	captive vs. free-living population	Birds lighter at 1 month, heavier than wild at 2 months	(Sepp *et al*., 2010)*
		house sparrow (*Passer domesticus*)	repeated measures: multiple timepoints	Weight loss on days 11-25, regained by day 35	Chapter 3*
		chukar partridge (*Alectoris chukar*)	repeated measures: multiple timepoints	Weight loss at day 1, partially regained at 5 and 9 days	(Dickens *et al.*, 2009b)*
	fish	skipjack tuna (*Katsuwonus pelamus*)	repeated measures: multiple timepoints	Weight loss at day 2, regained at 20 days	(Bourke *et al.*, 1987)

*data from this paper were used to generate Figure 2.2.

[1] No at-capture values – first measured at 2 months.

[2]Captive pups were rehabilitated after rescue.

[3] Slight weight loss from day 10 to day 60.

[4] Females did not reach at capture weight, but all spontaneously aborted or gave birth.

Table 2.2: Patterns of change in baseline and integrated glucocorticoids when wild animals are brought into captivity. This table does not include studies where the pattern was different in different seasons – those studies may be found in Table 2.6.

GC Pattern during adjustment to captivity	Species		Study design	Timeframe	How were free-living GCs established?	Sample type	Citation
No effect on GCs over captivity period	mammals	degu (*Octodon degus*)	captive vs. free-living populations	>1 year	free-living population	plasma (<2 min)	(Quispe *et al.*, 2014)*
		brushtail possums (*Trichosurus volpecula*) (♂ only)	repeated measures: multiple timepoints	20 weeks	none – first sample at week 1 of captivity	plasma (<5 min)	(Baker *et al.*, 1998)
		brushtail possums (*Trichosurus volpecula*) (♂)	repeated measures: multiple timepoints	up to 8 weeks	none – unclear when first sample was obtained	plasma (time not given)	(Begg *et al.*, 2004)
		harbor seal (*Phoca vitulina*) (juvenile)	captive vs. free-living populations	>4 weeks	free-living animals	plasma (wild: 60 min captive: <10 min)	(Trumble *et al.*, 2013)*
		tuco-tuco (*Ctenomys talarum*)[1]	repeated measures: multiple timepoints	30 days	at-capture measure	plasma (<3 min)	(Vera *et al.*, 2011)*
	birds	European starling (*Sturnus vulgaris*)	repeated measures; pre- vs post- captivity	4 weeks +	feather grown in the wild	feathers	Appendix I
		western screech owl (*Otus kennicottii*)	captive vs. free-living populations	>1 month	free-living animals	plasma (<5 min)	(Dufty and Belthoff, 1997)*
		house sparrow (*Passer domesticus*)	multiple timepoints; different individuals	up to 4 weeks	free-living animals	plasma (<3 min)	(Martin *et al.*, 2011)*
	reptiles	tuatara (*Sphenodon punctatus*) (♂ only)	captive vs. free-living populations	unknown	free-living population	plasma (<20 min)	(Tyrrell and Cree, 1994)*
		kutum (*Rutilus frisii kutum*)	captive vs. free-living populations	3 days	free-living population	plasma (<3 min)	(Nikoo *et al.*, 2010)*
GCs elevated in captivity	mammals	Canada lynx (*Lynx canadensis*)[2]	captive vs free-living populations	long term (unknown)[3]	free-living population	FGMs	(Fanson *et al.*, 2012)*
		spider monkey (*Ateles geoffroyi yucatanensis*)	captive vs free-living populations	long term (unknown)3	free-living population	FGMs	(Rangel-Negrin *et al.*, 2009)*

GC Pattern during adjustment to captivity	Species		Study design	Timeframe	How were free-living GCs established?	Sample type	Citation
		African wild dog (*Lycaon pictus*)	captive vs free-living populations	long term (unknown)3	free-living population	FGMs	(Van der Weyde *et al.*, 2016)*
		Grevy's zebra (*Equus grevyi*)	repeated measures: multiple timepoints	6 weeks	at-capture sample; free-living population	FGMs	(Franceschini *et al.*, 2008)*
		white rhinos (*Ceratotherium simum*)	repeated measures: multiple timepoints	75 days	at-capture sample	FGMs	(Linklater *et al.*, 2010)*
	birds	curve-billed thrasher (*Toxostoma curverostre*)	repeated measures: multiple timepoints	80 days	at-capture samples	plasma (<3 min)	(Fokidis and Deviche, 2011)*
		white-crowned sparrow (*Zonotrichia leucophrys*)	captive vs free-living populations	35 days	free-living population	plasma (<1 min)	(Marra *et al.*, 1995)*
		white-throated sparrow (*Zonotrichia albicollis*)	captive vs free-living populations	35 days	free-living population	plasma (<1 min)	(Marra *et al.*, 1995)*
		blackbirds (*Turdus merula*)	repeated measures; pre- vs post-captivity	22 days	at-capture sample	plasma (<3 min)	(Adams *et al.*, 2011)*
		house sparrow (*Passer domesticus*)	repeated measures: multiple timepoints	7 days	at-capture sample	plasma (<3 min)	(Fischer and Romero, 2016)*
		house sparrow (*Passer domesticus*)	repeated measures: multiple timepoints	7 days	at-capture sample	plasma (<3 min)	Chapter 5*
		house sparrow (*Passer domesticus*)	repeated measures; pre- vs post-captivity (multiple seasons)	5 days	at-capture sample	plasma (<3 min)	(Lattin *et al.*, 2012a)*
	reptiles	tuatara (*Sphenodon punctatus*) (♀ only)	captive vs. free-living populations	unknown	free-living population	plasma (<20 min)	(Tyrrell and Cree, 1994)*
		garter snake (*Thamnophis elegans*)	repeated measures: multiple timepoints	4 months	at-capture sample; free-living population	plasma (<10 min[4])	(Sparkman *et al.*, 2014)*
		tree lizard (*Urosaurus ornatus*)	multiple timepoints; different individuals	up to 3 weeks	at-capture samples	plasma (<1 min)	(Moore *et al.*, 1991)*

GC Pattern during adjustment to captivity	Species		Study design	Timeframe	How were free-living GCs established?	Sample type	Citation
		water snake (*Nerodia sipedon*)	repeated measures; pre- vs post-captivity	5-8 days	at-capture samples	plasma (<5 min)	(Sykes and Klukowski, 2009)*
		brown treesnake (*Boiga irregularis*)	multiple timepoints; different individuals	3 days	free-living population	plasma (<8 min)	(Mathies *et al*., 2001)*
	amphibians	cururu toad (*Rhinella icterica*)	repeated measures; pre- vs post-captivity	3 months	at-capture sample	plasma (<3 min)	(de Assis *et al.*, 2015)*
		green frog (*Rana esculenta*)	repeated measures: multiple timepoints (multiple seasons)	3 days	at-capture sample	plasma (<5 min)	(Zerani *et al.*, 1991)*
	fish	Coral reef fish (*Hemigymnus melapterus*)	captive vs. free-living populations	2.5 months	free-living population	plasma (<6 min)	(Grutter and Pankhurst, 2000)*
		wedge sole (*Dicologoglossa cuneate*) (juvenile)	multiple timepoints; different individuals	45 days	at-hatching samples	whole-body (time not given)	(Herrera *et al.*, 2016)*
GCs increase at-capture, then decrease to approach wild baseline	mammals	beluga whale (*Delphinapterus leucas*)	repeated measures: multiple timepoints	peak:1 day approach free-living by 4 days	at-capture sample and free-living population	plasma (time not given)	(St Aubin and Geraci, 1989)*
		Chacma baboon (*Papio ursinus*)	repeated measures: multiple timepoints	Peak: 4 weeks approach long-term captives by 7 weeks	None – used long term captives as baseline.	Plasma (time not given)	(Steyn, 1975)
		African green monkey (*Cercopithecus aethiops*)	multiple timepoints; different individuals	peak: 1 day approach free-living by: 2 days	free-living population	plasma (time not given)	(Suleman *et al.*, 2004)*
		mouse lemur (*Microcebus murinus*)	repeated measures: multiple timepoints	peak: 2 days approach free-living by: 4 days	at-capture sample	FGMs	(Hämäläinen *et al.*, 2014)*

GC Pattern during adjustment to captivity	Species		Study design	Timeframe	How were free-living GCs established?	Sample type	Citation
		Richardson's ground squirrel (*Urocitellus richardsonii*)	repeated measures: multiple timepoints	peak: 3-5 days approach free-living by: 6 days	at-capture sample	FGMs	(Hare *et al.*, 2014)*
		bottlenose dolphin (*Tursiops truncatus*)	repeated measures: multiple timepoints	peak: 1 day approach long-term captive by: 2 weeks	long-term captives	plasma (time not given)	(Orlov *et al.*, 1991)
	birds	house sparrow (*Passer domesticus*)	repeated measures: multiple timepoints	peak: day 7 approach free-living by: day 11	at-capture sample	plasma (<3 min)	Chapter 3*
		house sparrow (*Passer domesticus*)	repeated measures: multiple timepoints	peak: day 1-2 approach free-living by: 1 month	at-capture sample	plasma (<3 min)	(Kuhlman and Martin, 2010)*
		white-crowned sparrow (*Zonotrichia leucophrys*)	repeated measures: multiple timepoints	peak: day 1-2 approach free-living by: day 14	at-capture sample	plasma (time not given)	(Wingfield *et al*., 1982)*
	reptiles	skink (*Egernia whitii*)	repeated measures: multiple timepoints	peak: 1 day – 1 week approach free-living by: 4 weeks	at-capture sample	plasma (<1 min)	(Jones and Bell, 2004)*
	amphibians	water frog (*Rana esculenta*)	multiple timepoints; different individuals	peak: day 1 approach free-living by: day 7	free-living populations	plasma (<3 min)	(Gobbetti and Zerani, 1996)*
		cane toad (*Rhinella marina*)	repeated measures: multiple timepoints	peak: day 5 approach free-living by: day 12	at-capture sample	urine	(Narayan *et al*., 2011)*
		cane toad (*Rhinella marina*)	repeated measures: multiple timepoints	peak: day 4 approach free-living by: day 14	at-capture sample	urine	(Narayan *et al*., 2012)*
		Fijian ground frog (*Platymantis vitiana*)	repeated measures: multiple timepoints	peak: day 5	at-capture sample	urine	(Narayan and Hero, 2011)*

GC Pattern during adjustment to captivity	Species		Study design	Timeframe	How were free-living GCs established?	Sample type	Citation
				approach free-living by: day 25			
	fish	flounder (*Paralichthys orbignyanus*)	multiple timepoints; different individuals	peak: 1 hour approach free-living by: day 1	free-living animals	plasma (<7 min)	(Nestor Bolasina, 2011)*
		kahawai (*Arripis trutta*)	multiple timepoints; different individuals	peak: 2-3 hours approach free-living by: day 3	free-living animals	plasma (<4 min)	(Davidson *et al*., 1997)*
GCs lower in captivity	mammals	harbor porpoise (*Phocoena phocoena*)	captive vs. free-living populations	long term (unknown)	free-living population	plasma (time not given)	(Siebert *et al*., 2011)*
		Gilbert's potoroo (*Potorous gilbertii*)	captive vs. free-living populations	long term (unknown)	free-living population	FGMs	(Stead-Richardson *et al*., 2010)*
		harbor seal (*Phoca vitulina*)	repeated measures: multiple timepoints	long term (unknown)	free-living population	plasma (time not given)	(Gardiner and Hall, 1997)*
		black rhino (*Diceros bicornis*)[5]	repeated measures: multiple timepoints	60 days	at-capture sample	FGMs	(Linklater *et al*., 2010)*
		white whale (*Delphinapterus leucas*)	repeated measures: multiple timepoints	11 days	long-term captives	plasma (time not given)	(Orlov *et al*., 1991)
	birds	European starling (*Sturnus vulgaris*)	captive vs. free-living population	unknown	free-living population	FGMs	(Cyr and Romero, 2008)
		chukar partridge (*Alectoris chukar*)	repeated measures: multiple timepoints	9 days	at-capture sample	plasma (<3 min)	(Dickens *et al*., 2009b)*
High initial GCs decrease over capture period	mammals	rhesus macaques (Macaca mulatta)	repeated measures: multiple timepoints	decreased from day 1 to 1 year	none – first sample after unknown time in trap.	plasma (<50min)	(Lilly *et al*., 1999)
		brushtail possums (*Trichosurus volpecula*) (♀ only)	repeated measures: multiple timepoints	decreased from week 1 to 20	none – first sample at week 1 of captivity	plasma (<5 min)	(Baker *et al*., 1998)
		meadow vole (*Microtus pennsylvanicus*)	multiple timepoints; different individuals	decreased from day 1 to day 70	none – first sample at day 1	plasma (<1 min)	(Olsen and Seabloom, 1973)

GC Pattern during adjustment to captivity	Species		Study design	Timeframe	How were free-living GCs established?	Sample type	Citation
		vicuñas (*Vicugna vicugna*)	repeated measures: multiple timepoints	decreased from at-capture to day 12	none – first sample after stressful capture (time not given)	plasma (time not given)	(Bonacic and Macdonald, 2003)
		Eurasian otter (*Lutra lutra*)[6]	repeated measures: multiple timepoints	decreased from days 2-5 to days 5-10	none – first sample at day 2-5	plasma (time not given)	(Fernandez-Moran *et al*., 2004)
	birds	red knot (*Calidris canutus*)	repeated measures: multiple timepoints	decreased from first sample to 2 years	none – first sample at day 70	plasma (3-38 min)	(Piersma and Ramenofsky, 1998)
	fish	red gurnard (*Chelidonichthys kumu*)	repeated measures; pre- vs post-captivity (different durations)	decreased from first sample to 1 day	none – first sample after long line capture	plasma (<2 min)	(Clearwater and Pankhurst, 1997)
		snapper (*Pagrus auratus*)	multiple timepoints; different individuals	decreased form at-capture to day 2	none – first sample after long line capture	plasma (<10 min)	(Pankhurst and Sharples, 1992)
		sardine (*Sardina pilchardus*)	multiple timepoints; different individuals	decreased from at-capture to day 2	none – first sample after seine capture	plasma (~3 min)	(Marcalo *et al*., 2008)

*data from this paper are incorporated into Figure 2.3.

[1] Cortisol results only

[2] No difference in GCs in females pre-breeding – GCs elevated in both sexes during breeding season.

[3] Captive population may include some captive-raised individuals

[4] Blood sampling took longer in some samples

[5] GC spike in many animals during first 2 weeks, but then drops well below at capture levels.

[6] Some animals treated with long-acting neuroleptic, which had no effect on GC levels, so values were pooled.

Table 2.3: Patterns of change in stress-induced glucocorticoids and negative feedback with captivity in wild animals.

GC Pattern during adjustment to captivity	Species		Study design	Timeframe	How was free-living GCs established?	Sample type	Citation
No change in acute stress-induced GCs over captivity period	mammals	tuco-tuco (*Ctenomys talarum*)	captive vs. free-living populations	20 days	free-living population	Plasma (30 and 60 min)	(Vera *et al*., 2011)
	birds	curve-billed thrasher (*Toxostoma curverostre*)	repeated measures: multiple timepoints	80 days	at-capture samples	Plasma (30 min)	(Fokidis and Deviche, 2011)
		blackbirds (*Turdus merula*)	repeated measures; pre- vs post-captivity	22 days	at-capture sample	Plasma (30 and 60 min)	(Adams *et al*., 2011).
		western screech owl (*Otus kennicottii*)	captive vs. free-living populations	>1 month	free-living animals	Plasma (6-10 min)	(Dufty and Belthoff, 1997)
		house sparrow (*Passer domesticus*)[1]	repeated measures; pre- vs post-captivity	5 days	at-capture sample	Plasma (30)	(Lattin *et al*., 2012a)
		house sparrow (*Passer domesticus*)	multiple timepoints; different individuals	up to 1 month	at-capture sample	Plasma (60 min)	(Kuhlman and Martin, 2010)
		house sparrow (*Passer domesticus*)	repeated measures: multiple timepoints	7 days	at-capture sample	Plasma (30)	(Fischer and Romero, 2016)*
		house sparrow (*Passer domesticus*)	repeated measures: multiple timepoints	7 days	at-capture sample	Plasma (30)	Chapter 5*
		white-crowned sparrow (*Zonotrichia leucophrys*)[2]	repeated measures; multiple timepoints	up to 1 year	free-living population	Plasma (<30 min)	(Romero and Wingfield, 1999)
	fish	winter flounder (*Pseudopleuronectes americanus*)	repeated measures; multiple timepoints	up to 1 year	free-living population	Plasma (60 min)	(Plante *et al*., 2003)
Acute stress-induced GCs reduced in captivity	birds	chukar partridge (*Alectoris chukar*)	repeated measures: multiple timepoints	9 days	at-capture sample	Plasma (30 min)	(Dickens *et al*., 2009b)
		white-crowned sparrow (*Zonotrichia leucophrys*)[3]	repeated measures; multiple timepoints	up to 1 year	free-living population	Plasma (30 min)	(Romero and Wingfield, 1999)
Acute stress-induced GCs increased in captivity	mammals	degu (*Octodon degus*)	captive vs. free-living populations	>1 year	free-living population	Plasma (30 and 60 min)	(Quispe *et al*., 2014)

GC Pattern during adjustment to captivity	Species		Study design	Timeframe	How was free-living GCs established?	Sample type	Citation
	birds	white-crowned sparrow (*Zonotrichia leucophrys*)[4]	repeated measures; multiple timepoints	up to 1 year	free-living population	Plasma (30 min)	(Romero and Wingfield, 1999)
	reptiles	water snake (*Nerodia sipedon*)	repeated measures; pre- vs post-captivity	5-8 days	at-capture sample	Plasma (60 min)	(Sykes and Klukowski, 2009)
	amphibians	Eastern red-spotted newt (*Notophthalmus viridescens*)[5]	repeated measures; multiple timepoints	>1 year	free-living population	Plasma (30 min)	(Berner *et al*., 2013)
Negative feedback strength decreased with captivity, then increased	birds	chukar partridge (*Alectoris chukar*)	repeated measures: multiple timepoints	Neg. feedback reduced at day 5 recovered at day 9	at-capture sample	Plasma (90 min after DEX)	(Dickens *et al*., 2009b)
Negative feedback strength increased with captivity	birds	house sparrow (*Passer domesticus*)	repeated measures; pre- vs post-captivity	5 days	at-capture sample	Plasma 90 min after DEX)	(Lattin *et al.*, 2012a)

[1] SI GCs lower post captivity in early winter, but no change during any other time of year.
[2] Outside of breeding season and molt
[3] During the breeding season
[4] During the post-breeding/molting season
[5] SI GCs higher post captivity in pre-breeding and breeding season, not in winter.

Table 2.4: Changes in leukocytes during captivity. Timeframe refers to the longest duration of captivity measured. WBC=total white blood cells. H=heterophils. N=neutrophils. L=lymphocytes. n.c.=not calculated (in this case, a count or percentage of heterophils or neutrophils and lymphocytes was measured in the paper, but H or N:L ratio was not directly compared. Presence/direction of change in the ratio was inferred from patterns in leukocyte types.) ↑ or ↓ = higher or lower than free-living. – = no change from free-living. Data from all papers were used to construct Figure 2.4.

Species		Study design	How was free-living value established?	Timefra me	WBCs	H or N	L	H or N:L ratio	Citation
mammals	Spanish ibex (*Capra pyrenaica hispanica*)	repeated measures; multiple timepoints	At capture sample	14 months	↓	–	↓	↑ (n.c.)	(Peinado *et al*., 1995)
	rhesus macaques (Macaca mulatta)	repeated measures: multiple timepoints	At capture sample	1 year	↓	↓	↑	↓ (n.c.)	(Lilly *et al*., 1999)
	brushtailed possums (*Trichosurus vulpecula*)	repeated measures; multiple timepoints	None – first sample at week 1 of captivity	20 weeks	–			–	(Baker *et al*., 1998)
	beluga whale (*Delphinapterus leucas*)	repeated measures; multiple timepoints	At capture sample and free-living population	2.5 months	↑	↑	↓	↑ (n.c.)	(St Aubin and Geraci, 1989)
	brushtailed possums (*Trichosurus vulpecula*)	repeated measures; multiple timepoints (different housing conditions)	None – unclear when first sample was obtained	2 months	–	–	–	– (n.c.)	(Begg *et al*., 2004)
	howler monkey (*Alouatta caraya*)	repeated measures; pre- vs post-captivity	At-capture sample	2 months	–	–	–	– (n.c.)	(Sanchez-Sarmiento *et al.*, 2015)
	Steller sea lions (*Eumetiopias jubatus*)	repeated measures; pre- vs post-captivity	At-capture sample and free-living population	2 months	↓				(Mellish *et al*., 2006)
	black rhinoceros (*Diceros bicornis michaeli*)[1]	repeated measures; pre- vs post-captivity	None – first sample after stressful capture (up to 1 hour)	3-4 weeks		↑	↓	↑ (n.c.)	(Kock *et al*., 1999)
	vicuñas (*Vicugna vicugna*)[2]	repeated measures; multiple timepoints	At-capture sample[3]	12 days	–	–	–	–	(Bonacic and Macdonald, 2003)
birds	red knots (*Calidris canutus*)	captive vs. free-living population	Free-living population	~1 year	–	↓	–	↓ (n.c.)	(Buehler *et al*., 2008)
	ruff (*Philomachus pugnax*)	repeated measures; multiple timepoints	None – does not say when first sample taken relative to capture	1 year	–				(Piersma *et al*., 2000)

Species		Study design	How was free-living value established?	Timefra me	WBCs	H or N	L	H or N:L ratio	Citation
	greenfinches (*Chloris chloris*)	captive vs. free-living population	Free-living population	2 months	–	–	↑	↓	(Sepp *et al*., 2010)
	zebra finches (*Taeniopygia guttata*)	captive vs. free-living population	Free-living population	10 days	↓			↓	(Ewenson *et al*., 2001)
				2 months	–			↓	
	house sparrow (*Passer domesticus*)[4]	repeated measures: early- vs late-captivity	None – first sample 1-2 days in captivity	1 month	–	–	↓	↑ (n.c.)	(Kuhlman and Martin, 2010)
	herring gull (*Larus argentatus*)	repeated measures; multiple timepoints	at-capture sample	4 weeks	↑	↑	–	↑ (n.c.)	(Hoffman and Leighton, 1985)
	rufous-collared sparrows (*Zonotrichia capensis*)	captive vs. free-living population	Free-living population	2 weeks		↑	↓	↑	(Ruiz *et al*., 2002)
reptiles	garter snakes (*Thamnophis elegans*)	repeated measures; pre- vs post-captivity	At-capture sample and Free-living population	4 months				↑	(Sparkman *et al*., 2014)
amphibians	cururu toad (*Rhinella icterica*)	repeated measures; pre- vs post-captivity	At-capture sample	3 months	↑			–	(de Assis *et al*., 2015)
	Fijian ground frog (*Platymantis vitiana*)	repeated measures; multiple timepoints	At-capture sample	15 days		↑	↓	↑	(Narayan and Hero, 2011)
				25 days		–	–	–	
	mole salamanders (*Ambystoma talpoideum*)	repeated measures; pre- vs post-captivity	At-capture sample	10 days		–	↓	↑	(Davis and Maerz, 2008)
fish	kutum (*Rutilus frisii kutum*)	captive vs. free-living population	Free-living population	3 days	↑	↑	↓	↑ (n.c.)	(Nikoo *et al*., 2010)

[1] Pattern only seen in rhinos translocated from high to low (not high to high) elevation.
[2] Total WBCs and N:L ratio also compared to free-living wild populations of a similar species – there was no difference.
[3] Comparison to values collected in another study and species (llamas and alpacas).
[4] Circulating leukocytes and skin-infiltrating leukocytes were measured. See text for skin leukocyte patterns.

Table 2.5: Reproductive effects of captivity in wild animals. If multiple times of year were examined, only breeding season is included in this table.

Hormonal changes during adjustment to captivity	Species		Study design	Timeframe	Variable measured	How were free-living state established?	Citation
Reproductive capacity decreased in captivity	mammals	white rhino (*Ceratotherium simum*)	Repeated measures; multiple timepoints	75 days	Fecal T (males) and Progestin (females)	At-capture samples	(Linklater *et al*., 2010)
		black rhino (*Diceros bicornis*)	Repeated measures; multiple timepoints	60 days	Fecal T (males) and Progestin (females)	At-capture samples	(Linklater *et al*., 2010)
		mouse lemur (*Microcebus murinus*) (♀ only)	Pathology of dead captive animals	Variable – usually years in captivity	Histological examination of reproductive organs (follicular growth)	Reproductive pathology increased with captivity length	(Perret, 1982)
	birds	brown-headed cowbird (*Molothrus ater*) (♂ only)[1]	Captive vs. free-living population	6 months + 3 months photostimulation	Gonad size and plasma T	Free-living population	(Dufty and Wingfield, 1986)
		house sparrow (*Passer domesticus*) (♂)	Repeated measures; multiple timepoints	3 months	Sperm production, beak color, testes size	At-capture samples and free-living population	(Lombardo and Thorpe, 2009)
	reptiles	brown treesnakes (*Boiga irregularis*) (♂)	Captive vs. free-living population	4 to 8 weeks	Sexual maturity (testes development)	Free-living population	(Aldridge and Arackal, 2005)
		Anole lizard (*Anolis pulchellus*) (♀)	Multiple timepoints; different individuals	4 weeks	Plasma vitellogenin; ovary state[2]	Free-living population	(Morales and Sanchez, 1996)
		tree lizard (*Urosaurus ornatus*) (♂)	Repeated measures; multiple timepoints	3 weeks	Plasma T	At-capture samples	(Moore *et al*., 1991)
		snapping turtle (*Chelydra serpentina*)	Repeated measures; multiple timepoints	1 week	Plasma T[3]	At-capture sample	(Mahmoud *et al*., 1989)
	amphibians	water frog (*Rana esculenta*) (♂)	Repeated measures; multiple timepoints	2 weeks	Plasma T and E2	At capture samples	(Gobbetti and Zerani, 1996)
		water frog (*Rana esculenta*)	Repeated measures; multiple timepoints	3 days	Plasma T and E2[4]	At capture sample	(Zerani *et al*., 1991)
	fish	jack mackerel (*Trachurnus jabonicus*) (♀)	Captive vs. free-living population	1 year	Egg maturity, reproductive stage, gnrh gene expression[5]	Free-living population	(Imanaga *et al*., 2014)

Hormonal changes during adjustment to captivity	Species		Study design	Timeframe	Variable measured	How were free-living state established?	Citation
		electric fish (*Gnathonemus petersii*)	Repeated measures; multiple timepoints	37 days	Sex-specific behaviors, plasma T and 11KT (males)	At capture samples	(Landsman, 1993)
		sardine (*Sardina pilcardus*)	Captive vs free-living population	4 weeks	Gonadosomatic index	Free-living population	(Marcalo *et al.*, 2008)
		red gurnard (Chelidonichthys kumu) (♀)	Multiple timepoints; different individuals AND Repeated measures; multiple timepoints	4 days	Plasma T, E2, egg development	Free-living population	(Clearwater and Pankhurst, 1997)
No difference in reproductive capacity in captivity	mammals	armadillos (*Dasypus novemcinctus*) (♂)	Repeated measures; multiple timepoints	up to 3 years	Plasma T	Free-living population	(Czekala *et al.*, 1980)
	birds	white-crowned sparrows (*Zonotrichia leucophrys*)[6]	Repeated measures; multiple timepoints	up to day 20 or 33	Plasma LH, plasma T (males only)	At-capture sample	(Wingfield *et al.*, 1982)
	reptiles	striped plateau lizard (*Sceloporus virgaltus*) (♀)	Repeated measures; multiple timepoints	up to 3 months	Plasma P, T, and E2[7]	Free living population	(Weiss *et al.*, 2002)
		skink (*Egernia whitii*) (♂)	Repeated measures; multiple timepoints	4 weeks	Plasma T	At-capture samples	(Jones and Bell, 2004)
		brown treesnake (*Boiga irregularis*)	Multiple timepoints; different individuals	3 days	Plasma T and ovarian follicle development	Free-living population	(Mathies *et al.*, 2001)
	amphibians	water frog (*Rana esculenta*) (♀)	Repeated measures; multiple timepoints	2 weeks	Plasma T and E2[8]	At capture samples	(Gobbetti and Zerani, 1996)

[1] Different conditions tested – maximal testicular regrowth (long days + females) below wild, though in that group, T was the same as wild.
[2] Vitellogenin levels recovered by E2 use.
[3] T spikes during the first 24-48 hours of captivity, but decreases below at-capture levels.
[4] E2 spikes during first hours of captivity, but quickly decreases below at-capture levels.
[5] E2 higher in captive than wild.
[6] There was a transitory increase in LH at around week 1-3 that came back to at-capture levels in multiple experiments.
[7] T lower in captivity, but only after egg-laying.
[8] E2 spike in first 6 hours of captivity but then returns to at-capture levels.

Table 2.6: Seasonal effects of captivity. Arrows indicate direction of change in captive animals relative to an established free-living level. Captive and wild measurements were taken at the same time of year. GCs glucocorticoids; T testosterone; E2 estradiol

Hormone	Species		How was free-living GCs established?	Captivity duration	Pre-breeding	Breeding	Post-breeding/molt	Winter	Citation
baseline GCs	mammals	Canada lynx (*Lynx canadensis*) females	Free-living population	Long term (unknown)	–			↑	(Fanson *et al*., 2012)
		Canada lynx (*Lynx canadensis*) males	Free-living population	Long term (unknown)	↑			↑	(Fanson *et al*., 2012)
		Harbor seal (*Phoca vitulina*)	Free-living population	Long term (unknown)	↓	↓	↓		(Gardiner and Hall, 1997)
	birds	white-crowned sparrow (*Zonotrichia leucophrys gambelii*)	Free-living population	4 months at start	–	↓	↑	–	(Romero and Wingfield, 1999)
		house sparrow (*Passer domesticus*)	Same individual pre-capture	5 days	↑	–	–	–	(Lattin *et al*., 2012a)
	reptiles	Duvaucel's gecoks (*Hoplodactylus duvaucelli*)	Free-living population	>1 year at the start	–	↑		–	(Barry *et al*., 2010)
		tuatara (*Sphenodon punctatus*) (♀)	Free-living population	Unknown		↑		–	(Tyrrell and Cree, 1994)
		tuatara (*Sphenodon punctatus*) (♂)	Free-living population	Unknown		–		–	(Tyrrell and Cree, 1994)
	amphibians	eastern red-spotted newt (*Notophthalmus viridescens*)	Free-living population	2 months to >1 year	↑	↑		–	(Berner *et al*., 2013)
		green frog (*Rana esculenta*)	Same individual pre-capture	3 days	↑	↑	↑		(Zerani *et al*., 1991)
	fish	winter flounder (*Pseudopleuronectes americanus*) (juvenile)	Free-living population	2 months at start	↓[1]		–		(Plante *et al*., 2003)

Hormone	Species		How was free-living GCs established?	Captivity duration	Pre-breeding	Breeding	Post-breeding/molt	Winter	Citation
Acute stress GCs	birds	white-crowned sparrow (*Zonotrichia leucophrys gambelii*)	Free-living population	4 months at start	–	↓	↑	–	(Romero and Wingfield, 1999)
		house sparrow (*Passer domesticus*)	Same individual pre-capture	5 days	–	–	–	↓	(Lattin *et al.*, 2012a)
	amphibians	eastern red-spotted newt (*Notophthalmus viridescens*)	Free-living population	>1 year	↑	↑		–	(Berner *et al.*, 2013)
	fish	winter flounder (*Pseudopleuronectes americanus*) (juvenile)	Free-living population	2 months at start	–		–		(Plante et al., 2003)
T	mammals	armadillos (*Dasypus novemcinctus*)	Free-living population	2 weeks-3 years	–	–	–	–	(Czekala *et al.*, 1980)
	amphibians	green frog (*Rana esculenta*) (♀)	Same individual pre-capture	3 days	↓	–	–		(Zerani *et al.*, 1991)
		Amphibian: green frog (*Rana esculenta*) (♂)	Same individual pre-capture	3 days	↓	↓	↓		(Zerani *et al.*, 1991)
E2	amphibians	Amphibian: green frog (*Rana esculenta*) (♀)	Same individual pre-capture	3 days	↓	↓	↓		(Zerani *et al.*, 1991)
		Amphibian: green frog (*Rana esculenta*) (♂)	Same individual pre-capture	3 days	–	–	↓		(Zerani *et al.*, 1991)

[1] Delay in acquiring wild baseline.

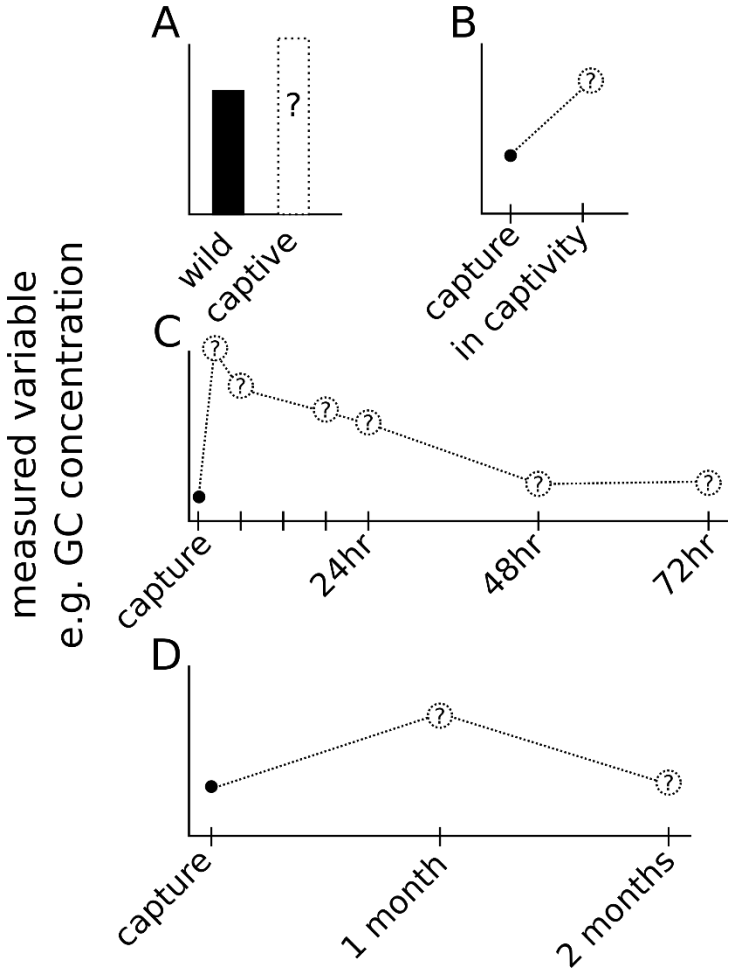

Figure 2.1 Examples of experimental designs to assess the effects of captivity on a physiological variable (e.g. GC concentration). A) Comparison of captive individuals to free-living populations. In some cases, the free-living samples were acquired at the same time that the study population was brought into captivity. In other designs, the free-living samples were taken from entirely different populations than the origin of the captive animals (e.g. comparing zoo-housed animals to wild conspecifics). B) Each individual measured immediately at capture and again after a period of captivity (days to months). C and D) Each individual measured immediately at capture and resampled at multiple timepoints. Some

studies focused on the first few days, with sampling points relatively close together (C). Other studies may not have taken another sample until several weeks after capture (D). Note that graphs represent typical a priori predictions, not actual data.

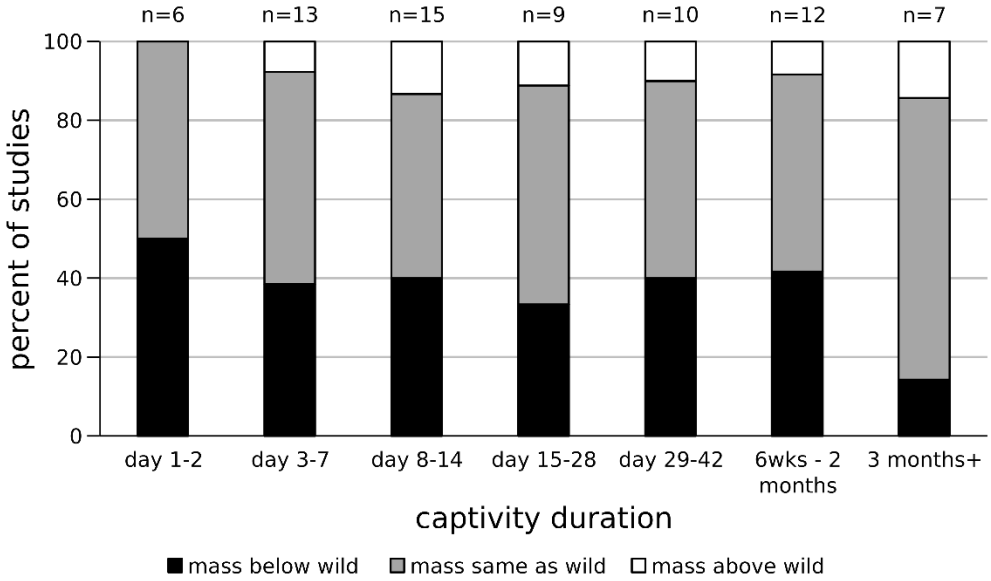

Figure 2.2 Weight change as a function of captivity duration. Data were collected from 31 studies listed in Table 2.1. The number of species that lost weight in captivity (relative to wild, free-living animals) decreased with captivity duration.

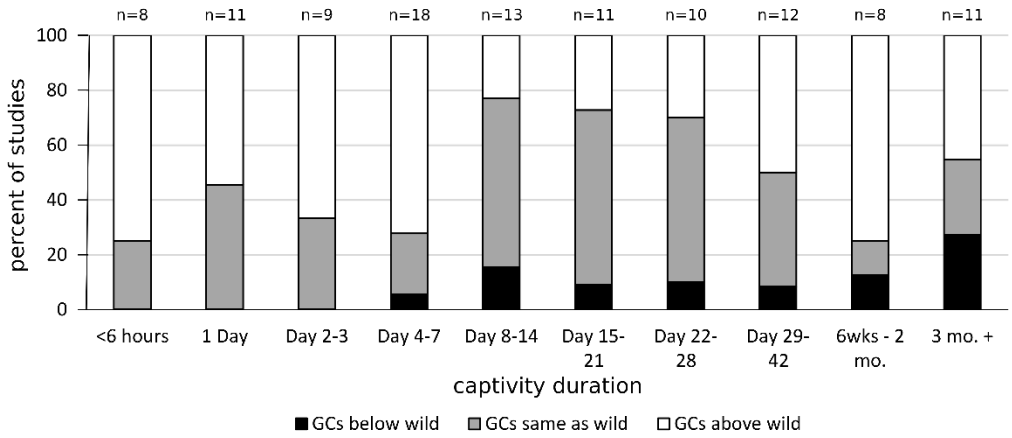

Figure 2.3 Change in baseline or integrated glucocorticoids as a function of captivity duration. Data were collected from the 47 studies listed in Table 2.3 that had a well-defined wild baseline value (i.e. plasma samples were collected within minutes of capture; fecal or urine samples were collected shortly after capture). This figure does not include studies with seasonal effects on the GC response to capture.

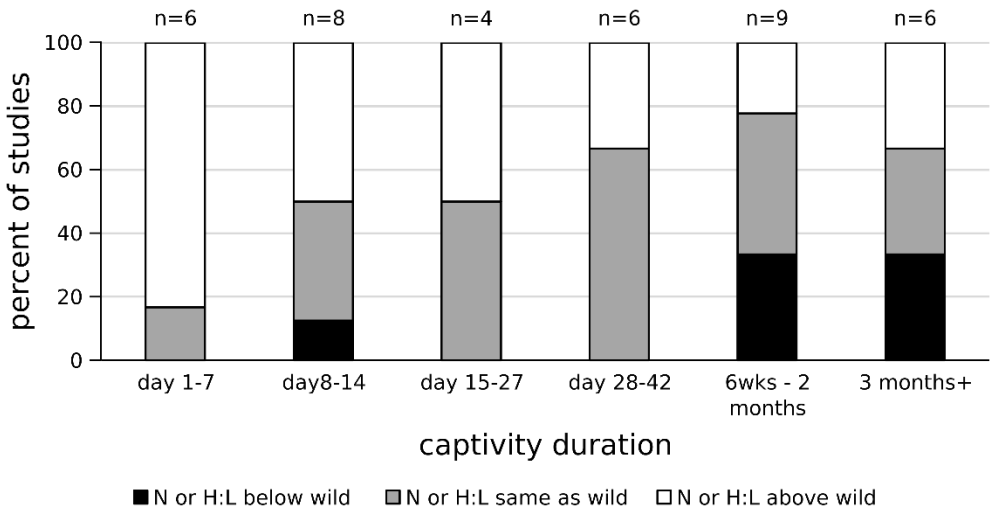

Figure 2.4 Changes in neutrophil or heterophil to lymphocyte ratio (N or H:L) in captivity as a function of time. Data were collected from 19 studies listed in Table 2.4. The percent of studies that recorded elevated N or H:L ratio in captivity decreased with the amount of time spent in captivity.

CHAPTER 3: CHRONIC STRESS AND THE INTRODUCTION TO CAPTIVITY: HOW WILD HOUSE SPARROWS ADJUST TO LABORATORY CONDITIONS.

Clare Parker Fischer, Jessica Wright-Lichter, and L. Michael Romero

3.1 Abstract

The conditions of captivity can cause chronic stress in wild animals. Newly-captured animals may experience weight loss, elevated glucocorticoid hormones, increased heart rate, increased resting adrenomedullary activation, and an altered heart rate response to acute stressors. As captivity conditions persist, chronic stress may decrease as animals adjust to the stressors of captivity. In this study, house sparrows (*Passer domesticus*) were captured from the wild, fitted with heart rate transmitters, and individually housed in an indoor bird facility. Mass, baseline corticosterone, resting heart rate, resting adrenomedullary activation, and the acute heart rate response to a sudden noise were measured over the course of the first 6 weeks of captivity. Birds lost weight during the first weeks of captivity which was regained by week 5. Baseline corticosterone peaked at day 7, decreased sharply by day 11, and continued to decrease throughout the 6 weeks. Although heart rate in the first 24 hours could not be collected, daytime heart rate decreased from day 1 through day 20, where it reached a stable plateau. Daytime heart rate variability decreased through the entire 6 weeks, which may indicate a gradual shift from sympathetic to parasympathetic nervous system regulation of heart rate. The acute heart rate response to a sudden noise lasted longer at day 6 than earlier or later in

captivity. In conclusion, the data indicate that the different physiological systems associated with chronic stress adjust to captivity over different timelines.

3.2 Introduction

Captivity can be a potent source of stress for newly captured wild animals. The conditions of captivity (e.g. confinement, artificial lighting, changes in diet, and the presence of and handling by humans) are unpredictable, uncontrollable stimuli that can activate the stress response systems (Morgan and Tromborg, 2007). During the initial introduction to captivity, wild birds may experience weight loss (e.g. Ruiz *et al*., 2002; Lattin *et al*., 2012a), high heart rate (Dickens and Romero, 2009; Fischer and Romero, 2016), elevated glucocorticoid hormones (e.g. Lattin *et al*., 2012a; Fokidis *et al*., 2011), and alterations to other systems, such as the immune (e.g. Buehler *et al*., 2008) and reproductive systems (e.g. Lombardo and Thorpe, 2009). Many animals are able to survive well in captivity long term, however, and the period of chronic stress may eventually by followed by acclimation, as the affected physiological systems return to normal or reach a new level appropriate to their new environment. We monitored newly-captured house sparrows (*Passer domesticus*) for 6 weeks to determine the timing of acclimation to captivity with a focus on baseline corticosterone (CORT; the primary glucocorticoid in birds) and the adrenomedullary system.

The stress response is adaptive; a mechanism to help vertebrates survive challenges to homeostasis (Romero and Wingfield, 2016). A noxious stimulus, such as a predator attack, stimulates first an adrenomedullary response (an increase in the catecholamine hormones epinephrine and norepinephrine leading to an

increase in heart rate) followed shortly by a hypothalamic-pituitary-adrenal (HPA) axis response (culminating in an increase in glucocorticoid hormones) (Sapolsky *et al*., 2000). These physiological systems work together to help the animal survive the crisis. However, when stressors are ongoing or repeated, the resulting chronic stress can be harmful (Romero *et al*., 2009). In captive conditions, wild birds may experience a loss in mass (Fokidis *et al*., 2011; Lattin *et al*., 2012a), an increase in baseline CORT (Adams *et al*., 2011; Fokidis *et al*., 2011; Lattin *et al*., 2012a), changes in behavior (Adams *et al*., 2011; Fokidis *et al*., 2011), and changes to heart rate and heart rate variability (HRV; a metric of sympathetic nervous system activity; see section 3.3.4 [Dickens and Romero, 2009; Fischer and Romero, 2016]). The HPA and adrenomedullary systems are independently regulated (Nephew *et al*., 2003) and therefore may require different lengths of time to acclimate to captive conditions. However, the two systems do influence one another; glucocorticoids act permissively on the action of epinephrine and norepinephrine and so activation of the HPA axis can result in a more effective adrenomedullary response (Sapolsky *et al*., 2000).

In previous studies, we found that house sparrows had much higher heart rates during the first week after capture than after 1 month in captivity (Fischer and Romero, 2016; see also Chapter 4). Presumably, sometime between day 7 and day 30 of captivity, house sparrows must experience a decrease in heart rate, but how long this decline lasts and whether it has stabilized by day 30 is unknown. This is in contrast to European starlings (*Sternums vulgaris*), the only other bird species to our knowledge where heart rate has been monitored during adjustment to captivity.

European starlings had high heart rates at capture that decreased over the first 48 hours to a steady plateau that was the same as long term captives (Dickens and Romero, 2009). Newly-captured house sparrows had a moderately reduced acute response to startle compared to one month captives (Fischer and Romero, 2016). This pattern was more extreme in European starlings, which had a nearly eliminated startle response for at least the first 10 days of captivity (Dickens and Romero, 2009). House sparrows also had elevated baseline CORT during the first 5-7 days of captivity (Fischer and Romero, 2016; Lattin *et al*., 2012a). This may decline towards wild levels, as in white-crowned sparrows (Wingfield *et al*., 1982), or it may remain elevated, as in the curve-billed thrasher (Fokidis *et al*., 2011). In many lab studies that use wild birds, two weeks to one month is considered sufficient time for the animals to adjust to captivity (e.g. Bókony *et al*., 2014; Lattin and Romero, 2014). However, to our knowledge it is unknown whether acclimation in the HPA system coincides with acclimation in the adrenomedullary system for any species. The timing of acclimation for house sparrows, a species that is frequently used in laboratory studies, is also unknown. To answer these questions, we repeatedly sampled mass, baseline CORT, activity, heart rate, HRV, and the acute heart rate response to startle in wild house sparrows during the first 6 weeks of captivity.

3.3 Methods

3.3.1 Experimental Design

10 house sparrows (4 females, 6 males) were captured on or near the Tufts University campus in Medford, MA. A group of 5 birds was caught on June 2, 2016 (Group A) and a second group of 5 on June 3 (Group B). On the day of capture (day

0), birds were transported in cloth bags to the laboratory, where they were anesthetized and surgically fitted with a heart rate transmitter harness (see section 3.3.2). Birds were kept in individual, darkened cages until they had recovered from surgery. Because of possible anesthesia effects, we began collecting heart rate approximately 24 hours after capture. After surgery, birds were moved to individual cages in an indoor bird facility with a 15-hour light: 9-hour dark cycle to approximate the natural photoperiod at this time of year. They were provided with *ad libitum* food and water. Each cage was fitted with a receiver plate to record heart rate data. Because we had 10 birds and only 7 receiver plates, three cages did not have receiver plates. We rotated three of the Group A birds and three of the Group B birds between cages with and without receiver plates, such that birds were in the cages with receiver plates on all days for which heart rate recording was scheduled. For example, heart rate was recorded for Group A birds from day 1 to day 2 (June 3-4, 2016). At the end of the recording period, we switched cages for three Group A birds and three Group B birds. Heart rate for Group B birds was then collected for June 4-5 (day 1-2 for that group). A summary of the experimental design is shown in Figure 3.1.

Birds were weighed on days 0 (immediately upon capture), 4, 11, 18, 25, 32, and 39. On day 0 (immediately upon capture), and every 3-4 days thereafter, a blood sample was taken (see Figure 3.1). For each sample, the alar vein was punctured and ~40µl blood was collected in a heparinized capillary tube. We collected samples within 3 minutes of capture on day 0, and within 3 minutes of entering the bird room on all other days. Within this time frame, an acute increase

in CORT has not started or has only just begun (Romero and Reed, 2005). Blood samples were kept on ice until processing, when they were centrifuged at ~1200g for 8 minutes (Centrific Model 225, Fisher Scientific, Pittsburgh, PA, USA). Plasma was collected and frozen at -20ºC until CORT concentrations could be analyzed (see section 3.3.3).

On days 1, 3, 5, 9, 12, 16, 20, 27, 34, and 41, the birds were caught and their heart rate transmitters were switched on with a magnetic switch. Their resting heart rate was then automatically recorded using DataScience's Acquisition program for three minutes every two hours for 24 hours. At every sampling interval, the receivers also recorded a unitless activity metric. The receiver plate contains three radio receivers. Any change in relative signal strength between the three receivers is interpreted as movement within the cage. A higher activity score indicates more movement within the sampling interval. Activity was analyzed on DataScience's Analysis software.

On days 2, 6, 13, 21, 28, 35, and 42, at the end of the resting heart rate sampling period, an acute heart rate response to startle was recorded. Heart rate was recorded for 5 minutes. Then the door to the bird room was suddenly opened and closed. Heart rate was recorded for another 10 minutes. Afterwards, the heart rate transmitters were magnetically switched off to conserve batteries and the 24-hour sampling was initiated for the alternate group of birds.

3.3.2 Heart rate transmitters

We used the 1.6g Data Sci International PhysioTel heart rate transmitters, (model #ETA-F10). These transmit to a receiver plate attached to the side of the

cage. Although the transmitters are designed to be implantable, we used the harness-mounted method described in Fischer and Romero (2016). In brief, the body of the transmitter was sewn into a waterproof fabric pouch which was secured to a 3D printed base with the electrodes exposed. One electrode 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 exposed ends of the electrodes were sewn to the muscle. Four straps made of 0.5cm wide satin ribbon were sewn to the four corners of the base. Two straps were passed around the bird's neck and two were threaded under the wings so the base fit snugly against the body like a backpack and completely hid the part of the electrodes that extended out of the skin. The straps were sewn together at the center of the animal's chest.

3.3.3 Corticosterone analysis

We determined CORT concentrations in each sample using radioimmunoassay following Wingfield *et al*. (1992). Samples were assayed in duplicate, and assay values corrected for individual recoveries following extraction. Detectability was 1.07 ng CORT/ml plasma, inter-assay coefficient of variation was 10.4%, and intra-assay coefficient of variation was 2%. Any samples with undetectably low CORT were assigned to the limit of detection for statistical analysis.

3.3.4 Heart rate and heart rate variability analysis

Heart rate data were analyzed 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 heart rate traces were carefully inspected for misplaced R wave detection.

HRV 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 ~230 heartbeats for each 3-minute sampling window. The trace was visually inspected to ensure accurate identification of R-waves and individual marks were adjusted as necessary. HRV is a unitless measure adjusted for heart rate, with high HRV indicating beats are more irregular and low HRV indicating that they are more regular. HRV 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 HRV indicates more parasympathetic and less sympathetic activity) (Cyr *et al*., 2009; Korte *et al*., 1999; Perini and Veicsteinas, 2003).

The heart rate response to startle was analyzed using Ponemah P3 Plus soft-ware. Heart rate was analyzed in 5 second bins, with t=0 being the time of the sudden noise. For graphical purposes, 30 second bins were used. Heart rate was

averaged over the 5-minute pre-startle period to get a baseline heart rate. For each startle response, we analyzed the maximum heart rate in the 60 seconds following the noise, the maximum heart rate relative to the baseline (maximum heart rate - baseline heart rate), and the time for the heart rate to return to within one standard deviation of the baseline (the duration of the startle response). If heart rate never returned to baseline, it was assigned a maximum value of 10 minutes. Some of the startle responses could not be analyzed. Either there was an issue with recording (e.g. the bird was too far from the receiver plate to give a clear reading) or R-waves could not be distinguished on the trace due to noise. Sample sizes for the startle analysis ranged from 5 to 9 per captivity day.

3.3.5 Statistical analysis

All statistical analyses were conducted in R version 3.1.3 (R Core Team, 2013). To analyze the effect of captivity day on our measured variables, we constructed linear mixed effects models. When there was a monotonic relationship between captivity day and a variable, captivity duration was treated as a continuous variable (i.e. heart rate, HRV, and maximum heart rate after a startle). However, when a variable appeared to increase with time but then decrease, or vice versa, captivity duration was treated as a discrete variable (i.e. weight, baseline CORT, activity, and duration of the heart rate response to a startle). Linear mixed effects models were constructed using the "lmer" function in the lme4 package (Bates *et al*., 2015). 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. When

captivity duration was treated as a discrete variable, we followed this with a multiple comparison test if warranted, using the "glht" function from the multcomp package (Hothorn *et al*., 2008). We did not test every pairwise combination of values, but rather looked only at comparisons to the first value to determine if the animals' physiological state was different than in the wild. An alpha of $p<0.05$ was used to determine significance.

For activity, heart rate, and HRV data, we removed data points where the birds had been disturbed (e.g. because of animal care). We then averaged values for each light and dark cycle. Our sampling periods always began and ended in midafternoon. We therefore averaged the daytime samples from one afternoon with those from the following morning, even though they were separated by a night. Therefore, samples collected on the afternoon of day 1 and the morning of day 2 were all averaged and called "day 1" samples for analysis. We 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 HRV, we constructed separate models for daytime and nighttime.

Daytime heart rate appeared to decline to a certain point and then level off at a plateau. We searched for a statistical changepoint in the data, where before a particular day, the data followed one pattern (i.e. decreasing over time), but after that day, it followed a different pattern (i.e. no change over time). To find the breakpoint, we created a "hockey stick/broken stick" linear model, with a discontinuity at a particular point in our data. We then found the point where the deviance of this broken stick model was minimized – this was the breakpoint. We

then created an lmer with a discontinuity at the breakpoint. We compared this to an lmer without the discontinuity using the Akaike Information Criterion (AIC) scores for the two models.

3.4 Results

3.4.1 Weight

There was a significant effect of captivity day on mass ($F_{6,54}$=4.00, p=0.002, Figure 3.2A). The birds lost weight over the first four weeks of captivity, but regained it during weeks five and six. A multiple comparisons post hoc analysis revealed that the birds were significantly lighter at days 11, 18, and 25 compared to their pre-captivity weight ($p<0.01$ for all), but by day 32, there was no difference from initial weight. At capture, birds weighed 27.0±0.69g (average ± standard error), which decreased to a minimum of 23.6±0.64g at day 18.

3.4.2 Baseline corticosterone

Baseline CORT at capture was quite low, and was undetectable in 9 out of 10 birds. Baseline CORT significantly varied across 6 weeks of captivity ($F_{12,107.4}$=4.06, $p<0.0001$), increasing markedly by the day 7 sample, then decreasing again (Figure 3.2B). A multiple comparisons test revealed that at day 4 and day 7, baseline CORT was significantly higher than at capture ($p<0.05$). Baseline CORT then decreased. A multiple comparisons test of day 7 against all other days found that baseline CORT was significantly higher at day 7 than at all other days tested ($p<0.05$ for all). We removed the first three sampling points and looked for a linear relationship between baseline CORT and captivity duration. We found that from day 11 through day 42, baseline CORT decreased with sampling

day ($F_{1,88.2}$=4.12, p=0.04). At days 14 and 18, baseline CORT was marginally significantly elevated above baseline (p<0.1), but did not otherwise differ from the at capture level. Although the later samples were not significantly different from the pre-captivity measure, they were still somewhat higher and much more variable. At capture, birds had an average of 1.2±0.2 ng/ml CORT, while at day 42, they had 4.8±2.1 ng/ml.

3.4.3 Heart rate

There was a significant effect of time of day (daytime vs. nighttime) on heart rate ($F_{1,836.2}$=189.71, p<0.0001). Therefore, we analyzed daytime and nighttime heart rate separately (Figure 3.3A). Daytime heart rate decreased with days in captivity ($F_{1,73.4}$=14.85, p=0.0002). However, it did not decrease linearly throughout the captivity period. Instead, it seemed to decrease and then plateau. We found the most likely breakpoint to be day 18. We therefore created an lmer with a discontinuity at day 18. We found the heart rate decreased from day 1 to day 18 ($F_{1,72.2}$=17.98, p<0.0001, effect size=-4.85 bpm/day), but there was no relationship between captivity day and heart rate after day 18 ($F_{1,72.1}$=0.41, p=0.5). The model with the discontinuity better explained the data than the model without (AIC for model with discontinuity: 935.8; AIC for continuous model: 944.5).

3.4.4 Heart rate variability

There was a significant circadian effect on heart rate variability ($F_{1,836.3}$=104.42, p=0.0004), with daytime HRV higher than nighttime values, so HRV was analyzed separately during the day and at night (Figure 3.3B). Daytime HRV significantly decreased over the course of the captivity period ($F_{1,73.9}$=6.81,

p=0.01, effect size=-0.015 per day). There was no effect of captivity day on nighttime HRV ($F_{1,72.3}$=1.00, p=0.3).

3.4.5 Activity

There was a circadian effect on activity ($F_{1,956.3}$=428.9, p<0.0001), with birds being more active during the day than at night. We analyzed daytime and nighttime activity separately (Figure 3.3C). There was an effect of day of captivity on daytime activity ($F_{9,79.0}$=2.31, p=0.02). In a multiple comparisons post-hoc analysis, there was more activity on day 9 and day 34 than on day 1 (p=0.05). At night, there was so little recorded activity that the data could not be statistically analyzed. However, there was little observed change in nighttime activity across the time in captivity.

3.4.6 Heart rate response to startle

Heart rate for the 5 minutes before and 10 minutes after a sudden noise was sampled periodically throughout the 6-week study period (Figure 3.4A). There was no effect of time in captivity on the maximum heart rate or the maximum heart rate relative to the pre-startle baseline (Absolute max: $F_{1,43.9}$=0.36, p=0.6; Max relative to baseline heart rate: $F_{1,44.5}$=0.09, p=0.8), Figure 3.4B). The duration of the startle response (i.e. the time for heart rate to return to within one standard deviation of baseline) changed over the course of the captivity period ($F_{6,38.7}$=4.29, p=0.002, Figure 3.4C). A Tukey's post-hoc analysis revealed that the Day 6 startle response lasted significantly longer than on days 2, 13, 21, 28, and 35 ($p \leq 0.02$ for all).

3.5 Discussion

Wild house sparrows newly introduced into captivity showed many symptoms of chronic stress that gradually reduced over the course of 6 weeks of acclimation to captivity. However, the acclimation process was asynchronous in different systems.

Baseline CORT was very low in at capture, and was undetectable in 90% of the birds. It then increased over the first week in captivity, reaching a peak of 25.5±3.6 ng/ml around day 7. This is comparable to levels of CORT after 30 minutes of restraint in a bag for this species, and is close to the maximum adrenal capacity (Lattin *et al*., 2012a; Romero *et al*., 2006). In several species, glucocorticoid hormones were higher during the beginning of captivity and then decreased over the first days and weeks as the animals acclimated to captive conditions (e.g. white-crowned sparrows, *Zonotrichia leucophrys*, [Wingfield *et al*., 1982]; skink, *Egernia whitii*, [Jones and Bell, 2004]; and cane toads, *Rhinella marina*, [Narayan *et al*., 2012]). In a previous study, house sparrows brought into captivity for 5 days had elevated baseline CORT, though the concentrations were still much less than what we observed, reaching levels of only ~3 ng/ml during the time of year comparable to our study (Lattin *et al*., 2012a). In our study, although CORT had increased to 12.4±3.8 by day 4, peak CORT levels were not reached until day 7. After the initial high peak of baseline CORT, glucocorticoid levels rapidly dropped. Four days after the peak, at day 11, baseline CORT had significantly decreased, though it was still elevated from the at-capture values, with 90% of samples having detectable CORT, and 4 animals having CORT in the range

of stress-induced CORT in other studies (>15 ng/ml). CORT continued to decrease over the course of the study period from day 11 to day 42.

By the end of the 6-week study period, baseline CORT was not significantly different from the concentrations at capture, though it was somewhat higher and more variable. In some bird species, baseline CORT is higher after a few weeks or months in captivity than in the wild, such as blackbirds, *Turdus merula* (Adams *et al*., 2011); white-crowned and white-throated sparrows, *Zonotrichia leucophrys* and *Z. albicollis* (Marra *et al*., 1995); and curve-billed thrashers, *Toxostoma curvrostre* (Fokidis *et al*., 2011). These studies suggest that captivity may cause permanent alterations in the physiology of some animals. However, captivity can also cause decreased baseline CORT, as in chukar partridges, *Alectoris chukar* (Dickens *et al*., 2009b) or CORT can return to at-capture levels, as in one study with white-crowned sparrows (Wingfield *et al*., 1982).

There was significant variation in daytime activity across the study period, with activity increasing to peak at day 9 and then decreasing again. Blackbirds had increasing activity over the first 22 days of captivity (Adams *et al*., 2011). Daytime activity showed a similar pattern to baseline CORT – increasing and then decreasing over a similar timeframe. CORT can affect locomotor behavior in birds. White-crowned sparrows increased their activity rapidly after exogenous treatment with CORT (Breuner *et al*., 1998), although long-term CORT administration tended to decrease activity unless birds were fasted (Astheimer *et al*., 1992). Other hormones in the HPA axis can also affect behavior. Corticotropin releasing factor (CRF) can cause an increase in locomotor behaviors (reviewed in Bale and Vale, 2004). High

levels of CRF on days 7-9 could result in both increased activity and increased baseline CORT.

The pattern of weight loss and regain did not occur simultaneously with the pattern in CORT. A period of weight loss is frequently seen when animals are brought into captivity, after which the mass is usually regained. For example, white-crowned sparrows (Wingfield *et al*., 1982); chukar (Dickens *et al*., 2009b); green-finches, *Chloris chloris* (Sepp *et al*., 2010); possums, *Trichosurus vulpecula* (Baker *et al*., 1998); and rabbits, *Oryctolagus cuniculus* (Calvete *et al*., 2005) all showed similar patterns of weight loss and regain. Weight loss can be explained by a shift in diet, but is also frequently attributed to glucocorticoids. CORT can cause break-down of protein during gluconeogenesis, leading to muscle wasting (Romero and Wingfield, 2016). The initial period of weight loss in captivity occurred in tandem with high levels of glucocorticoids. However, while CORT levels decreased at day 11 and continued to decrease, the birds did not regain weight until day 32. It is possible that the birds lost muscle mass during the initial period of captivity and that muscle was never regained, but was eventually replaced with fat.

The pattern in heart rate was unrelated to the patterns in glucocorticoids. Daytime heart rate was high (~600 bpm) on day 1, but decreased over the first 3 weeks of captivity. By day 20, it had plateaued at around 500 bpm, which is comparable to one month captives of this species in another study (Fischer and Romero, 2016). Nighttime heart rate was invariant in this species across the study period. In European starlings, heart rate decreased more rapidly and had reached a plateau by 48 hours after captivity (Dickens and Romero, 2009). We have not been able to

obtain resting heart rate from free living house sparrows, and so we do not know whether the heart rate plateau represents the normal state for these birds, or whether captivity has permanently altered the physiology of the animals. If the heart rate in the first weeks of captivity is high relative to free-living animals, or if the plateau is above the normal resting heart rate for this species, these animals may be at risk for cardiac damage, as high heart rate has been linked to death in captive birds (Domingo *et al*., 1991). If other species follow a similar trajectory in acclimating to captivity, they may bear similar risks and require a long adjustment period. Alternatively, the heart rate at capture may be close to the heart rate of free living birds, in which case, the decrease in heart rate may indicate a switch to a different physiological state in captivity. Heart rate correlates with body size in birds (Machida and Aohagi, 2001), so even without data from free-living house sparrows, we can estimate the heart rate by comparing to free-living birds in other similarly-sized passerines. Free-flying white eyed vireos (*Vireo griseus*, ~17g) had heart rates from 600-900 bpm (Bisson *et al*., 2009). Swainson's thrushes (*Catharus ustulatus*, ~30g) had resting heart rates were around 700 bpm (Bowlin *et al*., 2005). Therefore, a heart rate of 600 bpm is not abnormally high for a bird of this size, while a heart rate of 500 might be on the low end.

HRV did not show the expected pattern during adjustment to captivity. High HRV is typically interpreted as high parasympathetic nervous system control over heart rate relative to sympathetic control (Stauss, 2003; von Borell *et al.*, 2007). HRV has been demonstrated to decrease during an acute restraint stressor in European starlings, indicating higher sympathetic control during the stressor (Cyr *et al*.,

2009). We had expected HRV to increase over the study period, as it did in European starlings during their adjustment to captivity (Dickens and Romero, 2009). However, daytime HRV decreased over the remainder of the 6-week study period, although the decrease was not particularly strong. Nighttime HRV did not change, indicating that there was no overall shift in relative parasympathetic and sympathetic nervous system control at night. As there was no change in nighttime heart rate, this was not surprising. In other studies in this species, HRV has been shown to increase with duration in captivity for the first week (Fischer and Romero, 2016; and see Chapter 5). In house sparrows treated with saline for three days (a vehicle control for drug injection), daytime and nighttime HRV increased over the first week of captivity (Fischer and Romero, 2016). In sparrows treated daily with oil (a different vehicle control for drug injection), daytime and nighttime HRV also tended to increase during the first week in captivity (Chapter 5). Although in this study HRV decreased over the 6-week captivity period, there was a small increase in HRV from day 1 to day 5 that approached significance ($F_{1,17.5}$=3.28, p=0.09). These results would suggest that parasympathetic activity tends to increase in house sparrows during the first week of captivity, but then the parasympathetic contribution to heart rate decreases through the remainder of the 6 weeks. The process of parasympathetic withdrawal in control of heart rate is associated with cardiac disease in humans (Binkley *et al*., 1991; Bonaduce *et al*., 1997), and so may predict poor long-term cardiac health in these captive house sparrows. However, in the current study, the pattern is only seen during the day. Daytime HRV must be interpreted cautiously. HRV is most reliable as a metric of autonomous nervous

system activity in unmoving animals, as activity can cause higher HRV regardless of parasympathetic input (Stauss, 2003). Although daytime activity does not correlate with HRV (Figure 3.5), daytime HRV values still must be considered less reliable than nighttime HRV, when the animals are mostly still.

In a previous study in European starlings, the acute heart rate response to startle was greatly reduced during the first 10 days in captivity (Dickens and Romero, 2009). The startle response in house sparrows does not appear to be as strongly affected by captivity. We did not see a relationship with maximum heart rate during a startle response and time in captivity. However, we did find that the duration of the startle response was longer on day 6 than before or after that period. This corresponds to the period when baseline CORT was maximal. Baseline CORT acts permissively on the action of epinephrine and norepinephrine, and allows those hormones to function more effectively (Sapolsky *et al*., 2000). The increased length of startle response may therefore be explained by the high baseline CORT levels at this time period. This is supported by an experiment in which house sparrows were injected with mitotane to reduce CORT concentrations; the duration of the startle response was shorter in mitotane-treated birds than in oil-injected controls (Chapter 5).

It is worth noting that in this study, the house sparrows were adjusting not only to the many features of captivity that may be stressful, they also had the additional stressor of the heart rate transmitter harness. The birds were subjected to anesthesia and surgery on the day of capture and wore their harnesses through to the end of the experiment. Some of the birds required an additional brief surgery

later in the study to replace electrodes that they had pulled out. In vicunas, HPA adjustment to captivity was unaffected by an intensive handling procedure (shearing) (Bonacic and Macdonald, 2003). Untreated house sparrows had lower baseline concentrations of CORT at 5 days (~3 ng/ml, [Lattin *et al*., 2012a]) than what we measured at day 4 (~12 ng/ml). However, there is no way to measure the effect of the heart rate transmitter on heart rate parameters.

In conclusion, the adjustment to captivity was not coordinated in all physiological systems that we examined. Some appear to be related, such as the response to startle and baseline CORT, but others are largely independent of each other. This study thus supports other studies (e.g. Bonacic and Macdonald, 2003) that found that changes in mass, the HPA axis, the adrenomedullary system, the immune system, and the reproductive system are not necessarily synchronous. Perhaps unsurprisingly, captivity disrupts numerous physiological systems, and acclimating to captive conditions appears to require different time frames in those different physiological systems. House sparrows may require more than 4 weeks for the different aspects of their physiology to stabilize.

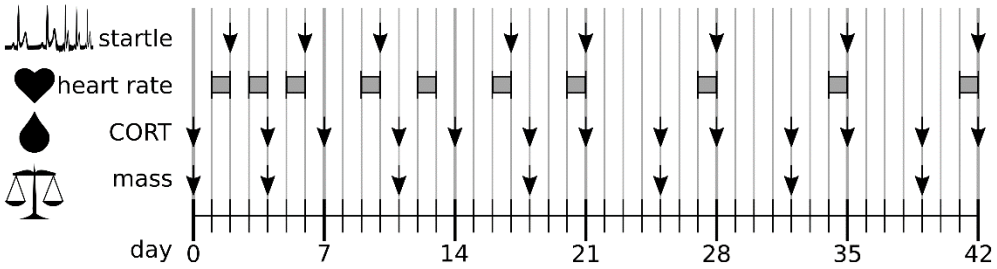

Figure 3.1 Timeline of experimental protocols for long term captivity experiment. 5 house sparrows were caught on June 2, 2016 and 5 were caught on June 3. Day 0 refers to the day of capture for both groups. Blood samples for CORT analysis were taken in the morning. Heart rate (as well as HRV and activity) was sampled for 3 minutes every 2 hours beginning in the midafternoon of one day and continuing through midafternoon of the following day. The acute heart rate response to a sudden noise (startle) was recorded after the heart rate samples were completed. Heart rate was recorded for 5 minutes, the door to the bird facility was opened and suddenly closed, and heart rate was recorded for a further 10 minutes.

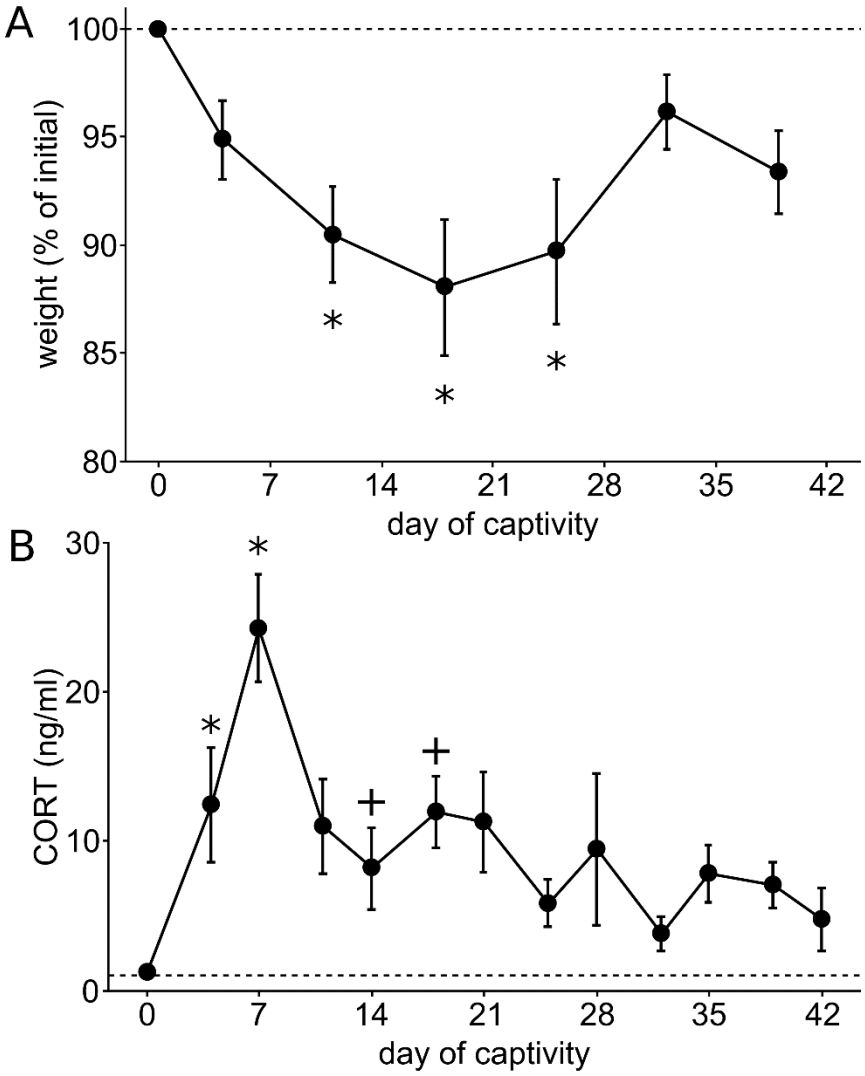

Figure 3.2 Weight and corticosterone in house sparrows during adjustment to captivity. A) Birds lost and then regained weight over six weeks in captivity. 100% was defined as weight at original capture. Birds were significantly lighter on days 11, 18, and 25 compared to at capture. They had regained weight to by day 32. B) Baseline CORT increased and then decreased over six weeks of captivity. On day 7, baseline CORT was significantly higher than at capture or on day 11 onward. N=10. Error bars represent mean ± standard error. * indicates significant difference from the at-capture measurements ($p<0.05$). + indicates marginally significant difference from the at-capture measurements ($p<0.1$).

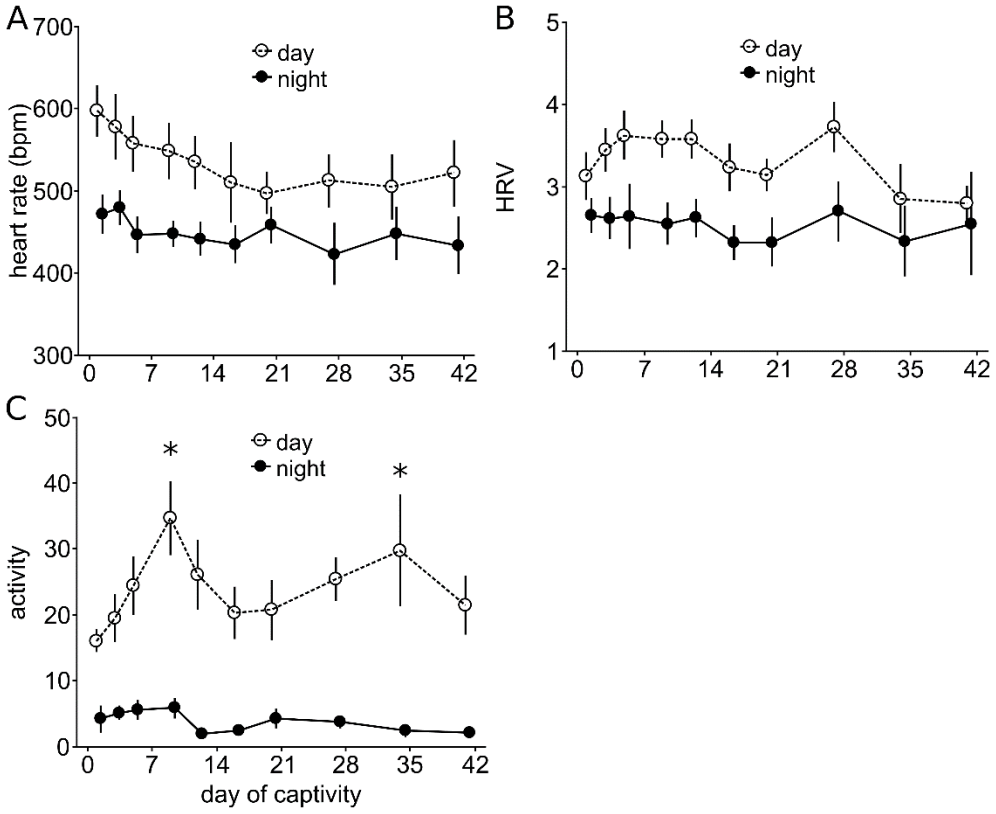

Figure 3.3 Heart rate and heart rate variability in house sparrows during adjustment to captivity. A) Daytime heart rate decreased from day 1 to day 18, then reached a plateau. Nighttime heart rate was invariant across the study period. B) Daytime HRV tended to increase across the study period. Nighttime HRV does not change with captivity day. C) Daytime activity was higher at day 9 than at day 1. Nighttime activity could not be statistically evaluated, but remained low throughout the study period. N=10. Error bars represent mean ± standard error. * indicates significant difference from day 1 ($p<0.05$).

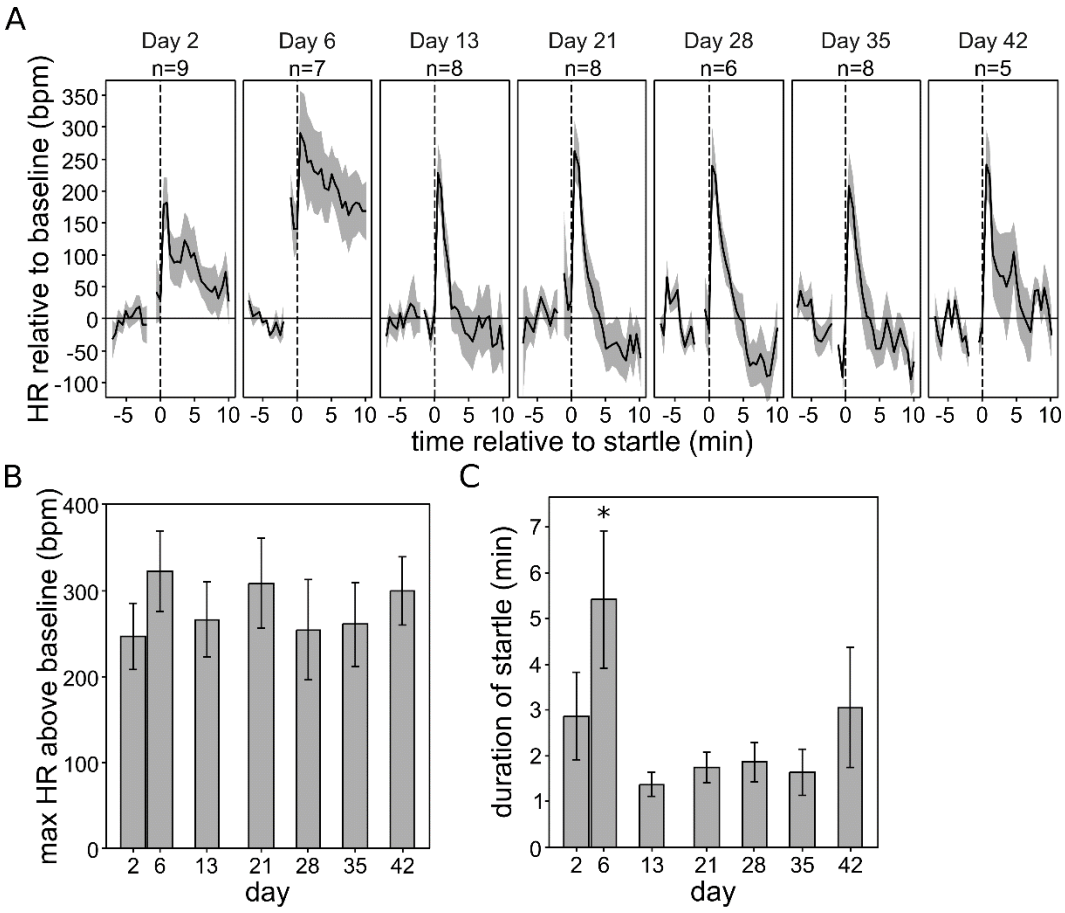

Figure 3.4 Heart rate response to a sudden noise during adjustment to captivity in house sparrows. A) Heart rate from the 5 minutes before a noise and the 10 minutes after it. Heart rate (HR) is relative to the pre-noise baseline. The dotted line indicates the sudden noise. The solid line and shaded gray area indicates mean HR ± 1 standard error. B) Maximum HR above the pre-noise baseline does not change with day of captivity. Error bars represent mean ± standard error. C) Duration of the startle response varies with day of captivity. Error bars represent mean ± standard error. * indicates significant difference from day 2 ($p<0.05$).

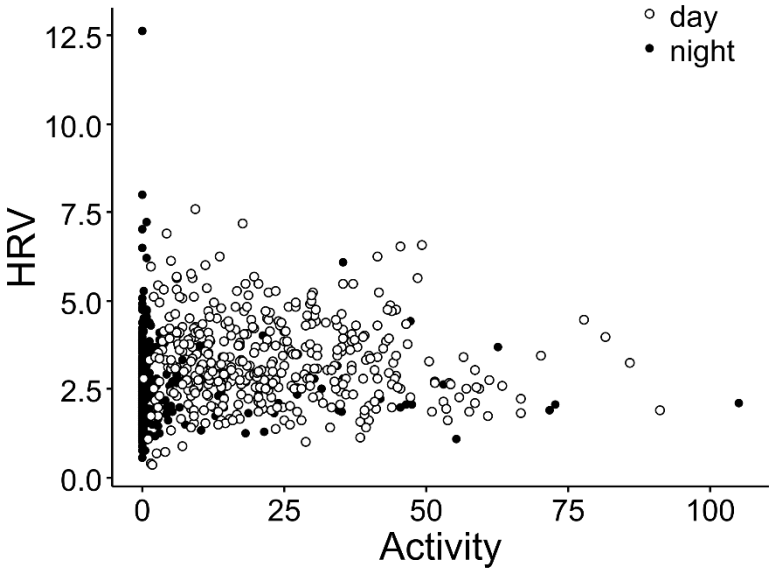

Figure 3.5 Activity does not correlate with heart rate variability in captive house sparrows. During the day, there is no relationship between activity and HRV ($F_{1,419}$=1.61, p=0.2). There is also no relationship at night ($F_{1,359}$=0.03, p=0.9).

CHAPTER 4: THE USE OF ALPHA- OR BETA-BLOCKERS TO AMELIORATE THE CHRONIC STRESS OF CAPTIVITY IN THE HOUSE SPARROW

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4.1 Abstract

When wild animals are brought into captivity for the first time, they frequently develop chronic stress symptoms. Animals can develop glucocorticoid dysregulation or changes in the sympathetic nervous system over the course of the first week in captivity. By blocking the action of epinephrine and norepinephrine using alpha- or beta-blockers, we hoped to reduce the degree of chronic stress symptoms exhibited by newly-captured house sparrows. We measured corticosterone, heart rate, and heart rate variability in 24 house sparrows (*Passer domesticus*) over the first week of captivity. The birds were treated with either saline, propranolol (a beta-blocker), or phentolamine (an alpha-blocker) for the first three days of captivity. We also compared newly-captured animals to animals which had been held in captivity for one month. During the first week of captivity, baseline corticosterone increased, but that increase was blocked by propranolol.

Heart rate was not different between the treatment groups, but it was higher during the first week than after one month in captivity. Sympathetic nervous system activity (as measured by heart rate variability) decreased over the first week of captivity, but was not affected by treatment. Beta-blockers, but not alpha-blockers, may help improve some symptoms of chronic stress in newly captured animals.

4.2 Introduction

When wild animals are brought into captivity, they are faced with circumstances unlike any they have experienced before. Confinement, artificial light conditions, altered diet, the presence of and handling by humans, and other factors contribute to unpredictable and uncontrollable living conditions (sources of stress in captivity reviewed in Morgan and Tromborg, 2007). Not surprisingly, chronic stress develops in wild animals of many species when they are brought into captivity (eg. Adams *et al*., 2011; Cabezas *et al*., 2007; Dickens *et al*., 2009b; Lattin *et al*., 2012a; Terio *et al*., 2004). Chronic stress occurs when stressors are ongoing or repeated and the physiological systems that are normally important for surviving and recovering from negative events become dysregulated and begin to cause problems. Although the effects of captivity on glucocorticoid hormones are relatively well documented in birds (Adams *et al*., 2011; Dickens *et al*., 2009b; Lattin *et al*., 2012a, Wingfield *et al.* 1982), less is known about the catecholamine side of the response. When animals overproduce epinephrine (E) and norepinephrine (NE) during the first few days of captivity, this may cause the production of these hormones to become dysregulated. They may produce too much E or NE at rest, resulting in a too-fast heart rate, or too little E or NE when it is

needed. Poorly regulated E and NE production could potentially cause negative health outcomes. By temporarily blocking the action of E and NE using receptor antagonists, we expected to reduce chronic stress symptoms in captive house sparrows. Not only will this study help to uncover the role of E and NE in developing chronic stress, it may provide useful tools for reducing chronic stress in birds brought into captivity for conservation or research.

There are two major hormonal systems in play during the stress response – the release of glucocorticoids (in birds, corticosterone, CORT) and the release of catecholamine hormones (E and NE) resulting in an increase of sympathetic nervous system (SNS) activation. In an acute stress response, CORT binds to receptors throughout the body, resulting in a shift in energy use away from reproduction, growth, and other long term investments and towards immediate survival and recovery (reviewed in Sapolsky *et al*., 2000). The release of E and NE from the adrenal medulla results in a very rapid increase in heart rate (the startle response), as well as changes in the respiratory, vascular, and digestive systems. The two systems are independently regulated in birds (Dickens *et al*., 2006; Nephew and Romero, 2003) but they do interact. CORT increases catecholamine secretion in birds (Mahata and Ghosh, 1991; Zachariasen and Newcomer, 1974) and increases responsiveness to catecholamines (reviewed in Sapolsky *et al*., 2000). E and NE stimulate the release of CORT by triggering the release of ACTH from the pituitary (mammals: Bugajski *et al*., 1995; Mezey *et al*., 1983; birds: Rees *et al.*, 1985).

The role of CORT in acute and chronic stress has been extensively studied in wild animals in both field and lab conditions. Chronic stress typically results in (and is often defined by) changes in CORT regulation following repeated exposures to a stressor. In many bird species, captivity has been found to cause changes in glucocorticoid concentrations, though the direction of change is species specific (Adams *et al*., 2011; Dickens *et al*., 2009b; Lattin *et al*., 2012a). However, the relationship between catecholamine hormones and chronic stress has been relatively understudied in wild birds. Dysregulation of the SNS may occur following high E/NE signaling during the first few days of captivity. Dickens and Romero (2009) documented elevated heart rate, elevated SNS activity, and a drastically reduced startle response in European starlings during the first days of captivity. A potentially diminished startle response during the transition to captivity has been reported in other animals. For example, newly-captured bighorn sheep had lower plasma concentrations of E and NE during an acute stressor than sheep raised in captivity (Coburn *et al*., 2010). Captive harbor porpoises also had lower plasma E and NE after being netted and sampled than free-living porpoises (Siebert *et al*., 2011). However, we do not know if an impaired startle response is a general response to captivity amongst many species.

In this study, we developed a new harness mounted system for recording heart rate in house sparrows, a much smaller (~27 gram) species than the European starling. We then chemically blocked catecholamine receptors during the first few days of captivity to assess the role of E and NE in developing chronic stress symptoms. There are two major types of E/NE receptors, alpha and beta receptors,

which are present in a variety of tissues, including the central nervous system. Alpha receptors are present in the vascular system and are responsible for constriction of smooth muscle, such as that found in peripheral blood vessels (Ahlquist, 1948, Minneman *et al*., 1981). Beta receptors are present on the heart and smooth muscle. They cause an increase in heart rate, as well as relaxation of smooth muscle, such as that found in bronchi (Ahlquist, 1948, Minneman *et al*., 1981). We tested the effects of blocking either alpha receptors (using 3 mg/kg phentolamine injected intramuscularly in the pectoralis) or beta receptors (using 3 mg/kg intramuscular propranolol) in house sparrows during the first week of captivity.

We expected that the chronic stress of captivity would cause the following changes in the physiology of newly captured house sparrows: 1) Lattin and colleagues (2012a) previously documented the effects of captivity on mass and glucocorticoid concentrations in house sparrows. We expected to replicate their results and see weight loss, increased baseline CORT, and increased CORT negative feedback after 1 week in captivity. 2) Dickens and Romero (2009) documented changes in heart rate over the first week of captivity in European starlings. We expected that house sparrows would similarly show high heart rate that would decrease over the first week. 3) A high heart rate can be due to increased SNS activity (and therefore higher concentrations of E and NE). However, the parasympathetic nervous system (PNS) also regulates heart rate; a high heart rate could alternatively indicate less PNS activity. We can tease this apart by examining heart rate variability (HRV). The PNS causes the heart rate to vary with every

breath cycle; variation caused by the SNS occurs on a longer timescale (Stauss, 2003). Therefore, by comparing the beat-to-beat intervals on a short timescale, we can determine how much influence there is of the PNS relative to the SNS (high HRV indicates more PNS and less SNS activity) (Cyr *et al*., 2009; Korte *et al*., 1999; Perini and Veicsteinas, 2003). Dickens and Romero (2009) found that newly captured European starlings had low HRV, which increased over the course of the first week. We expected to see a similar pattern in house sparrows. 4) Finally, Dickens and Romero (2009) documented a drastically reduced startle response in newly captured European starlings. We expected house sparrows to have a similarly suppressed startle response. Our predictions for the SNS were based on studies in European starlings, the only previous studies to our knowledge of heart rate in newly captive passerines. However, house sparrows and European starlings do not have identical corticosterone responses to chronic stress (Lattin *et al*., 2012a; Rich and Romero, 2005). It was reasonable to expect that house sparrows would also show somewhat different heart rate changes in response to captivity.

In addition to replicating previous studies on chronic captivity stress, we designed our study to determine how E and NE impacted the development of chronic stress symptoms and whether these symptoms could be ameliorated by blocking the adrenergic receptors. We hypothesized that blocking the adrenergic receptors would speed up acclimation to captivity. When comparing propranol-treated, phentolamine-treated and saline-treated animals, we predicted that: 1) propranolol- and phentolamine-treated birds would show a more rapid decrease in resting heart rate over the course of the first week in captivity than saline-treated

birds, 2) propranolol- and phentolamine-treated birds would show a more rapid increase in HRV over the course of the first week than saline-treated birds, and 3) propranolol- and phentolamine-treated birds would show a more robust startle response after 7 days compared to saline-treated birds. If blockage of E/NE reduces all chronic stress symptoms, not only those related to the cardiovascular system, we would expect treated birds to have a reduced baseline CORT compared to saline-treated birds, as well as potential differences in negative feedback in the CORT response and weight loss.

4.3 Methods

4.3.1 Drug validations and one month in captivity heart rate

Eight house sparrows were captured in Medford, MA and held in captivity for four weeks. After this period of acclimation, the birds were fitted with heart rate transmitter harnesses (see section 4.3.4). Resting heart rate and heart rate variability was recorded for 3 minutes every 2 hours for 3 days while the birds were left undisturbed except for normal animal care. These birds were also used to test the acute effect of propranolol, phentolamine, and saline on heart rate. Heart rate was collected for 10 minutes before injection with saline, propranolol, or phentolamine and for 15 minutes after the experimenter had left the room (total time of disturbance <5 minutes). The birds were divided into two groups haphazardly. One group were injected with saline, then phentolamine, then propranolol. The other group received propranolol, then phentolamine, then saline. They were given at least 4 hours for their heart rate to recover to baseline between treatments. Based on the pharmacological halflife of phentolamine in mice (50 min, Kerger *et al*.,

1988) and propranolol in rats (40 min, Lemmer *et al*., 1985), we expected the drugs to be effectively cleared from the system by this point. These captivity-acclimated animals were also tested for their startle response. Their heart rate was recorded for 10 minutes, the door of the bird room was suddenly opened and slammed shut, and heart rate was recorded for a further 10 minutes.

4.3.2 Experimental design

House sparrows were captured in Medford, MA between December 1, 2014 and June 30, 2015. 24 animals were used in the final experiments, 8 in each treatment group. Immediately at capture a series of blood samples was taken for CORT analysis (see section 4.3.3). The birds were fitted with a harness-mounted heart rate transmitter device within 3 hours of capture (see section 4.3.4). They were then transferred to individual cages in an animal facility on a 13L:11D light cycle. Birds were assigned at capture to one of three groups – saline, propranolol, and phentolamine. On day 0 (the day of capture), day 1, and day 2, the birds were injected intramuscularly once per day with either saline, 3 mg/kg propranolol, or 3 mg/kg phentolamine. Propranolol and phentolamine were dissolved in saline at a concentration of 5 mg/mL; initial bird weight was used to calculate the injection volume for both treatments and the saline control (eg. 15μL for a 25g bird). Birds were held in captivity for one week. Heart rate was automatically sampled for 3 minutes every 2 hours. On day 6, another series of blood samples was taken for CORT. On day 1 (before their daily injection) and day 7, the birds' startle response was measured. Heart rate was recorded for 10 minutes before the startle. At t=0,

the door to the room was suddenly opened and slammed closed. Heart rate was recorded for a further 10 minutes.

All experiments complied with Association for Assessment of Laboratory Animal Care guidelines and were approved by the Tufts Institutional Animal Care and Use Committee.

4.3.3 Plasma sampling and corticosterone analysis

On days 0 and 6, a series of blood samples was taken. A baseline sample was collected within 3 minutes of the bird being captured or the researcher entering the bird room. 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, an artificial glucocorticoid that stimulates negative feedback (Lattin *et al*., 2012a). 90 minutes after injection, a final blood sample was collected. For each sample, the alar vein was punctured and ~40µl blood was collected in a heparinized capillary tube. All blood samples were stored on ice and centrifuged at 1200g 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 (RIA) following Wingfield *et al*. (1992). Samples were assayed in duplicate, and assay values corrected for individual recoveries following extraction. Detectability was 1 ng CORT/ml plasma, inter-assay coefficient of variation was 28%, and intra-assay coefficient of variation was 4%.

4.3.4 Heart rate transmitter harnesses

We used the Data Sci International PhysioTel ETA-F10 model of heart rate transmitter. These transmitters measure 19x13x6mm and weigh 1.6 grams. They transmit on an AM radio frequency to a receiver plate attached to the side of the cage. They are designed to be fully implantable. Seven of the sparrows (1 phentolamine, 3 saline, 3 propranolol) were implanted with transmitters following Nephew and Romero (2003). However, these small birds did not tolerate the surgery as well as the more robust European starlings and the mortality rate was unacceptably high. (50% mortality: 8 out of 16 birds implanted with this method died from complications with the surgery. Data from the 8th surviving bird with an implanted transmitter could not be collected due to a weather emergency.) Therefore, we designed a new backpack-style harness mount for the transmitters following Small *et al*. (2004).

The base of the harness was 3D printed with lightweight plastic (Figure 4.1). The harness base has a gently curved surface to hold the transmitter, four holes on the corners for the ribbon straps, and a hole in the center to thread the electrodes through. The transmitter and the excess length of electrodes were sewn into a small waterproof fabric pouch with the ends of the electrodes (~3 cm) threaded through a small hole. We sewed 4 lengths of 0.5 cm wide satin ribbon (~3 cm each) to the corners of the base and secured the transmitter pouch to the ribbons with the electrodes passed through the center hole. The ends of the ribbons were melted slightly to prevent fraying. The electrodes were then ready to be implanted.

The sparrows were anesthetized with 4.5% isoflurane (Piramel Healthcare, Morpeth, Northumberland, UK) with an oxygen fllow rate of 0.8 L/min using a

vaporizer (Vet Equip, Livermoore, CA, USA). Once asleep, anesthesia was maintained at about 2.5%. At the beginning of surgery, birds were injected intramuscularly with 1mg/kg carprofen (brand name Rimadyl, Zoetis, Kalamazoo, MI, USA) as an analgesic. The top two ribbons of the harness were brought around the animal's neck and sewn in place in a V in the middle of the bird's chest. The bird was arranged on its chest and the surgical sites were disinfected using iodine and alcohol. A small incision (5mm) was made just to the left of the dorsal midline, in an area free from feather tracts. Another incision (5mm) was made at the cervico-scapular junction. Using blunt forceps, the skin was dissected away from the muscle between the two incisions so the forceps could pass through. We then threaded the first electrode under the skin in a cranial direction. The electrode was sewn to the muscle using 4-0 synthetic monofilament suture (Ethicon, Sommerville, NJ, USA). Another surgical site was prepared on the caudo-dorsal region near the ilium. A small incision was made at the posterior site, and blunt forceps were again used to dissect the skin from the muscle between the ilium and the dorsal midline. The second electrode was then pulled through under the skin and sutured to the muscle as previously described. Wounds were closed with suture and sealed with VetBond (3M Animal Care Products, St. Paul, MN, USA). The harness was then settled in place with the electrodes completely hidden under the harness base. Exact placement of the electrodes made little difference so long as one electrode was anterior to the heart and one was posterior, so the electrical potential could be measured across the heart.

The final two ribbons of the harness were passed under the wings and sewn together with the neck straps in the center of the animal's chest. We monitored the birds until they recovered from the anaesthetic. Recovery from anesthesia was uneventful. We found that a slightly tighter harness was better than one with any slack – if the straps were fairly snug, the bird was much less likely to get tangled or to be able to gain access to the electrodes or the straps. We used the harness mounted transmitter for 17 birds in the experiments (7 phentolamine, 5 saline, 5 propranolol). However, one bird in each treatment group had a failed transmitter, so only CORT data were collected for these animals.

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

Heart rate was recorded automatically using DataScience's Aquisition program. Beginning in the evening of day 0 (after 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. Samples were discarded when the animals had been disturbed within 45 minutes of sampling (eg. because of the caretakers, startle response sampling, or moving other animals in and out of the facility). When the program sampled heart rate, it simultaneously took a measurement of activity. The receiver plates contain within them three separate radio receivers. Any change in the relative signal strength between the three receivers is interpreted as a change in position of the animal, and is recorded as “activity.” This is a unitless metric that correlates with degree of movement in the cage.

Heart rate data were analyzed 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. A unitless metric of activity was recorded by the HR transmitters due to changes in position relative to the receiver plate.

4.3.6 Heart rate variability analysis

HRV 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 150-200 heartbeats for each 3 minute sampling window. The trace was visually inspected to ensure accurate identification of R-waves and individual marks were adjusted as necessary. The number of marks requiring manual adjustment depended on the individual trace and ranged from 0-30% of R-waves. HRV is a unitless measure adjusted for heart rate, with high HRV indicating beats are more irregular (and thus the heart is primarily under PNS control) and low HRV indicating that they are more regular (thus the heart is under SNS control).

4.3.7 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 of R (Bates *et al*., 2015). 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 normality, we used the "qqp" function from the car package in R (Fox and Weisberg, 2011), which generates theoretical quantile-quantile plots to compare our data to a normal distribution, with 95% confidence interval lines. We considered our data to be normally distributed when the majority of observations fell within the expected range.

To test for the acute effect of propranolol, phentolamine, or saline injection on heart rate, we measured 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 integrated heart rate. This was followed with a Tukey's multiple comparison test on finding significance. We tested for an effect of transmitter surgery (harness mounted vs implanted) on resting heart rate, activity, or HRV (day and night analyzed separately). We also confirmed that transmitter surgery type had no effect on baseline CORT, stress induced CORT, or the strength of negative feedback after dexamethasone injection.

Baseline CORT, stress induced CORT, and strength of negative feedback were analyzed in separate models. Baseline CORT was very skewed due to many samples being below the limit of detection of the assay. We used non-parametric Kruskal-Wallis tests to look for differences between CORT concentration at day 0 vs day 6 (treatment groups analyzed together and separately). We also tested for differences between treatment groups at capture and after one week. The strength

of negative feedback in the HPA axis was calculated as the percentage decrease from stress induced CORT 90 minutes after a dexamethasone injection. We confirmed that stress induced CORT and strength of negative feedback were normally distributed by comparison to theoretical q-q plots for normality. We tested for the effect of treatment, experiment day, and their interaction on stress induced CORT and negative feedback strength.

Resting heart rate, activity, and heart rate variability were all analyzed in the same way. For visual simplicity, we averaged values across each day and night for each bird and used these averages for graphing the data. For the analysis, however, we used all sampling points. First, we confirmed that there were circadian patterns in these variables by looking for an effect of day vs. night. We analyzed daytime and nighttime separately on finding circadian rhythms in all variables. We confirmed that the data were normally distributed by comparison to theoretical q-q plots. Some data were not normally distributed and were transformed as follows. Daytime heart rate data was negatively skewed, so was normalized by squaring. The transformed daytime heart rate data was still non normal, with a kurtosis of -1.13 (a "flattened" distribution). However, linear mixed models are robust against kurtosis violations, even when they are sensitive to skewness violations (Arnau *et al.*, 2013). HRV and daytime activity data were positively skewed and were normalized by natural log transformation. After transformation, HRV and daytime activity data were slightly negatively skewed (respectively, -0.25 and -0.41). With this degree of skew, our analysis method is robust for the HRV analysis, but there is an increased risk of Type 1 error for the activity data (Arnau *et al.*, 2013). For

each day or night, each bird had up to six measurements (taken every 2 hours). There was very little activity at night (635 of 799 nighttime datapoints had activity ≤ 2), so these data were not analyzed statistically. Final models included experiment day, treatment, and their interaction. We then compared newly-captured birds to birds held in captivity for one month, lumping all treatments when there was no treatment effect.

We analyzed two variables for the startle response: maximum heart rate after the startle and integrated heart rate for the first 10 minutes after the startle (area under the curve). Both maximum heart rate and integrated heart rate were normally distributed as confirmed by comparison to theoretical q-q plots for normality. Linear mixed models for the effects of experiment day, treatment, and their interaction were run on these variables. We also compared maximum and integrated heart rate at day 1, day 7, and after 1 month (all treatments lumped), followed by Tukey's post hoc comparisons as warranted.

4.4 Results

4.4.1 Drug and harness-mounted transmitter validations

Propranolol, phentolamine and saline were injected into 8 animals that had been living in captivity for >28 days. Heart rate was measured for 10 minutes before the disturbance and 15 minutes after the experimenter had left the room after injecting all birds. The injection procedure caused an increase in heart rate in all animals (Figure 4.2A). Integrated heart rate after injection was significantly different between treatment groups ($F_{2,13.2}$=6.74, p=0.01, Figure 4.2B). Integrated heart rate was lower after propranolol treatment than after phentolamine or saline

(Tukey's post hoc analysis: propranolol vs phentolamine: z=3.22, p=0.004; propranolol vs saline: z=-3.10, p=0.005; phentolamine vs saline: z=0.02, p=1).

There was no difference in daytime or nighttime heart rate between implanted vs. harness mounted heart rate transmitters (Day: $F_{1,20.2}$=0.52, p=0.5; Night: $F_{1,20.2}$=1.73, p=0.20). There was no difference in daytime or nighttime heart rate variability between implanted vs. harness mounted transmitters (Day: $F_{1,20.1}$=0.02, p=0.9; Night: $F_{1,20.3}$=0.33, p=0.6). One week post capture, there was no difference in baseline CORT (Kruskal-Wallis χ^2=0.78, df=1, p=0.4), stress induced CORT ($F_{1,22}$=1.03, p=0.32), or the strength of negative feedback after a dexamethasone challenge ($F_{1,22}$=0.71, p=0.4). Daytime activity was higher with the harness mounted transmitters compared to implanted ($F_{1,19.9}$=4.18, p=0.05). However, given the lack of difference in all other physiological variables and the small number of implanted transmitters, no further distinction was made between transmitter placement and data were combined for all further analyses.

4.4.2 Weight

During the first week of captivity, 87.5% (21 of 24) of the animals lost weight. Weight loss was not significantly different between treatment groups ($F_{2,21}$=1.91, p=0.17). Birds lost on average 11% of their starting mass over the course of one week.

4.4.3 Corticosterone

Baseline CORT was low, and many samples were below the limit of detection (28% of samples), so non-parametric Kruskal-Wallis tests were used. Baseline CORT was significantly higher at day 6 compared to day 0 when

treatments were lumped (Kruskal-Wallis χ^2=10.87, df=1, p=0.001, Figure 4.3A). There was no effect of treatment on day 0 (χ^2=2.87, df=2, p=0.2) or day 6 (χ^2=3.36, df=2, p=0.2). We also looked for a difference between day 0 and day 6 in baseline CORT in each treatment group separately. Baseline CORT increased between day 0 and day 6 in saline- and phentolamine-treated birds (respectively: χ^2=6.55, df=1, p=0.01; χ^2=4.98, df=1, p=0.03), but not in propranolol-treated birds (χ^2=1.38, df=1, p=0.2).

Stress induced CORT was not affected by experiment day, treatment, or their interaction (Experiment Day: $F_{2,21}$=2.23, p=0.2; Treatment: $F_{2,21}$=0.39, p=0.7; Interaction: $F_{2,21}$=0.16, p=0.9, Figure 4.3B). There was also no effect of experiment day, treatment, or their interaction on the strength of negative feedback after dexamethasone (Experiment day: $F_{1,21}$=0.24, p=0.6; Treatment: $F_{2,21}$=0.11, p=0.9; Interaction: $F_{2,21}$=0.01, p=1, Figure 4.3C).

4.4.4 Heart rate

During the first week of captivity, resting heart rate was higher during the day than at night ($F_{1,1390.3}$=683.0, p<0.00001, Figure 4.4). In birds that had been held in captivity for one month, we also saw a circadian pattern in resting heart rate ($F_{1,455.1}$=15.86, p<0.0001, Figure 4.4). We therefore analyzed daytime and nighttime heart rate separately. Daytime heart rate was negatively skewed, so was squared for analysis. Daytime heart rate did not change over the course of the first 7 days and did not differ by treatment (Treatment: $F_{2,25.0}$=0.07, p=0.9; Experiment Day: $F_{1,584.2}$=0.31, p=0.6; Interaction: $F_{2,584.5}$=0.79, p=0.5). We compared daytime heart rate between newly captive birds (all treatments lumped) and birds held in

captivity for one month. Heart rate was significantly higher in newly captured birds compared to birds held in captivity for one week ($F_{1,28}$=13.77, p=0.001). Nighttime heart rate decreased over the course of the first week of captivity, but there was no treatment effect and no interaction effect (Experiment Day: $F_{1,764.9}$=4.51, p=0.03; Treatment: $F_{2,21.3}$=0.17, p=0.84; Interaction: $F_{2,764.9}$=1.0, p=0.4). There was no difference in nighttime heart rate between the newly captive birds (all treatment groups lumped) and birds that had been in captivity for one month ($F_{1,28}$=2.74, p=0.1).

4.4.5 Activity

Activity is a unitless metric derived from the heart rate transmitters. We found a strong circadian rhythm in activity, with daytime activity much higher than nighttime ($F_{1,1357.2}$=1630.3, p<0.00001, Figure 4.5). This same pattern was seen in birds held in captivity for one month ($F_{1,449.1}$=466.15, p<0.00001, Figure 4.5). We analyzed daytime and nighttime activity separately. Daytime activity data was positively skewed and so was log transformed for analysis. During the day, there was no effect of experiment day, treatment, or their interaction (Experiment Day: $F_{1,550.2}$=1.11, p=0.3; Treatment: $F_{2,30.4}$=1.01, p=0.4; Interaction: $F_{2,551.7}$=0.01, p=1). There was no difference in activity level between newly captured birds (all treatments lumped) and one month captives ($F_{1,28}$=0.03, p=0.9). Nighttime data was skewed such that it could not be normalized. There was little activity at night (79% of activity values measured were less than 2), and there was no apparent pattern with treatment or day.

4.4.6 Heart rate variability

HRV data was positively skewed and therefore log transformed before analysis. During the first week of captivity, HRV was higher during the day than at night ($F_{1,1322.9}$=33.35, p<0.00001). This pattern also held in birds held in captivity for one month ($F_{1,448}$=57.12, p<0.00001). We therefore analyzed daytime and nighttime HRV separately. There was an effect of experiment day on daytime HRV, as well as an interaction effect, but no effect of treatment (Experiment Day: $F_{1,526.5}$=6.66, p=0.01; Treatment: $F_{2,25.9}$=0.17, p=0.8; Interaction: $F_{2,527.6}$=3.87, p=0.02, Figure 4.6A). Because of the interaction effect, we looked for an effect of experiment day on each treatment separately. Daytime HRV significantly increased over time in the saline and propranolol groups (Saline: $F_{1,207.2}$=5.74, p=0.02; Propranolol: $F_{1,144}$=3.77, p=0.05). However, there was no effect of experimental day in the phentolamine group ($F_{1,176.4}$=1.37, p=0.2). We compared daytime HRV during the first week of captivity (all treatment groups lumped) to one month captives and found no difference ($F_{1,27.9}$=1.12, p=0.3).

At night, HRV increased over time ($F_{1,756.2}$=61.65, p<0.00001, Figure 4.6B). There was no effect of treatment but there was a significant interaction between treatment and experiment day (Treatment: $F_{2,23.6}$=1.03, p=0.4; Interaction: $F_{2,755.9}$=10.97, p<0.00001). Because of the interaction effect, we tested the effect of experiment day on each treatment group separately. Nighttime HRV significantly increased over the first week of captivity in the saline and phentolamine groups (Saline: $F_{1,275.9}$=56.1, p<0.00001; Phentolamine: $F_{1,263.1}$=4.02, p=0.05). There was a marginally significant trend towards increasing HRV in the propranolol group ($F_{1,216.8}$=3.72, p=0.06). We compared nighttime HRV during the first week of

captivity (all treatment groups lumped) to one month captives and found no difference ($F_{1,28}$=0.16, p=0.7).

4.4.7 Startle response

A startle response was measured after one day in captivity (one injection had been received 18-24 hours earlier) and after 7 days in captivity (three injections had been received, with the last one being 4 days previous) (Figure 4.7A). There was no overall difference in maximum heart rate between day 1 and day 7, and no effect of treatment (Experiment Day: $F_{1,15.3}$=0.31, p=0.6; Treatment: $F_{2,17.4}$=0.03, p=1, Figure 4.7B). However, there was a marginally significant interaction effect ($F_{2,15.2}$=3.00, p=0.08). We therefore compared day 1 to day 7 for each treatment group separately. We found that propranolol-treated birds had significantly higher maximum heart rate at day 7 compared to day 1 ($F_{1,5.3}$=10.49, p=0.02), but maximum heart rate did not change between day 1 and day 7 in saline- or phentolamine-treated birds (Saline: $F_{1,4.3}$=1.51, p=0.28; Phentolamine: $F_{1,5.4}$=0.86, p=0.39). We then compared maximum heart rate a day 1, day 7, and after 1 month (treatment groups lumped) and found no effect of duration in captivity ($F_{2,29.0}$=3.24, p=0.05). Maximum heart rate at one month was significantly higher than at day 1 or day 7 (Tukey's post hoc analysis: one month vs. day 1: z=2.31, p=0.05; one month vs. day 7: z=2.51, p=0.03; day 1 vs day 7: z=-0.33, p=0.9).

Integrated heart rate was calculated for the 10 minutes following the startle. There was no difference in integrated heart rate day 1 vs day 7, no effect of treatment, and no interaction effect (Experiment day: $F_{1,13.7}$=1.33, p=0.3; Treatment: $F_{2,17.1}$=1.72, p=0.2; Interaction: ($F_{2,13.6}$=0.91, p=0.4, Figure 4.7C). We

compared integrated heart rate at day 1, day 7 and after 1 month (all treatment groups lumped), and found no effect ($F_{2,26.7}$=0.43, p=0.66).

4.5 Discussion

4.5.1 Corticosterone effects of chronic stress

The direction, intensity, and timing of the changes caused by chronic stress depends on the species and the type of stressor (Dickens and Romero, 2013). However, a change in glucocorticoid regulation is typically seen during chronic stress. Glucocorticoid concentrations have been demonstrated to change when wild animals are first brought into captivity in mammals (Cabezas *et al*., 2007; Franceschini *et al*., 2008; Terio *et al*., 2004), birds (Adams *et al*., 2011; Dickens *et al*., 2009b; Lattin *et al*., 2012a), reptiles (Jones and Bell, 2004), and amphibians (Narayan *et al*., 2011). The effects of captivity on the HPA axis in house sparrows at different times of year was previously reported by Lattin and colleagues (2012a). They found that captivity affects CORT production differently during different life history stages, but during most or all seasons, birds lost weight, baseline CORT increased, and the strength of negative feedback in the CORT response increased.

Consistent with Lattin and colleagues' findings, the birds in our study lost weight and we saw an increase in baseline CORT concentrations in saline- and phentolamine-treated birds. Following propranolol treatment, baseline CORT did not increase, so one symptom of chronic stress was reduced. Propranolol-treated birds may have had a less dysregulated HPA axis due to their treatment. The pituitary normally releases ACTH when it receives a signal of CRF from the hypothalamus. However, CRF is not the only molecule that causes ACTH

production; among other secretagogues, E and NE can both stimulate ACTH production (Bugajski *et al*., 1995; Mezey *et al*., 1983). During chronic stress, other ACTH secretagogues may be as important as CRF because CRF production would be shut down by negative feedback of CORT on the hypothalamus. If E and NE are blocked from acting at the pituitary, less ACTH and therefore less CORT would be secreted. During the first few days of a chronic stressor, this reduction of CORT production, even if temporary, may help prevent or at least delay the dysregulation of the HPA axis that leads to higher baseline CORT. Propranolol, but not phentolamine, prevents the increase in baseline CORT. This suggests that beta receptors in the pituitary are responsible for stimulating ACTH production, as has been found in rats (Bugajski *et al*., 1995; Mezey *et al*., 1983) and chickens (Rees *et al.*, 1985). However, the potential of propranolol to reduce HPA symptoms of chronic stress should not be overstated, as there was no overall treatment effect on baseline CORT on day 6.

4.5.2 Sympathetic nervous system effects of chronic stress in house sparrows

The effects of chronic captivity stress on heart rate in house sparrows has not been previously documented. Using our heart rate transmitter equipment, it is not possible to get heart rate data from a bird that is not currently in captivity – the bird must remain within about 0.3m of the receiver plate. We therefore compared heart rate data from newly captured birds with birds that had been held in captivity for more than a month. These birds have presumably acclimated to the conditions of captivity. However, we have no way of knowing whether this represents a

physiology similar to wild birds, or if captivity permanently alters the physiology of these animals.

Beta blockers typically reduce the heart rate response to stressors in both mammals (Ballard-Croft and Horton, 2002) and birds (Cyr *et al*., 2009). In our validation tests, propranolol caused a decrease in integrated heart rate over the 15 minutes following injection compared to phentolamine or saline. Phentolamine did not cause an acute change in heart rate. It is important to recognize that the treatments we administered were very transient. Propranolol has a pharmacological halflife of about 40 minutes in rats (Lemmer *et al*., 1985). Phentolamine's halflife in mice is around 50 minutes (Kerger *et al*., 1988). Therefore, a three day treatment of these drugs administered once per day is a very mild intervention, probably resulting in moderately decreased SNS activity for at most a few hours per day.

During the first week of captivity, newly captured birds had much higher daytime heart rates than the one month captives. This suggests that sometime between the first week and one month of captivity, heart rate decreases. In a previous study on European starlings newly brought into captivity, heart rate was elevated compared to long term captives only for the first 24 hours (Dickens and Romero, 2009). These data cannot be explained by changes in the animals' activity levels, as there was no difference in activity between newly captive birds and one month captives. We cannot isolate which factor or factors cause the high heart rate in the first week, but the birds presumably can gradually adapt to captive conditions.

HRV was no different when comparing recent captives to one month captives. This is in contrast to previous work in the European starling, where HRV

was low during the first 48 hours of captivity, gradually increasing towards the same level as long term captives (Dickens and Romero, 2009). Although we saw no difference in HRV between newly captured birds and one month captives, HRV did increase over the course of the first week during both day and night. This indicates increasing PNS or decreasing SNS activity over time, as we would expect to see in animals that are acclimating to the conditions of captivity. Low HRV over a short timescale has been associated with cardiac disease in humans (Stauss, 2003) and has been used as an indicator of poor welfare in other animals (von Borell *et al*., 2007). HRV can provide more information about the state of an animal than heart rate alone. For example, layer hens from high feather plucking lines had only a slight elevation in resting heart rate compared to low-plucking lines but they showed substantially reduced HRV (Korte *et al*., 1998). Therefore, we expect that the health of the animals increases over the course of the first week. Even though heart rate remains high, increasing HRV may mean the birds experience less stress as they acclimate to captivity.

The circadian pattern in HRV, with higher HRV during the day, which was present in both recent captives and long term captives, was unexpected. This indicates higher parasympathetic activity and lower sympathetic activity during the day than at night, which is counter-intuitive. A possible explanation may be that daytime HRV does not represent a resting animal. If the birds were moving around during the sampling period, their heart rate might be somewhat more irregular due to movement, not due to parasympathetic control. The higher variability would then be explained by greater activity during the day, rather than by the natural rhythm

of the PNS. Nighttime HRV is more likely to represent resting heart rate, without the influence of activity, as activity at night was generally quite low.

Phentolamine-treated birds differed from saline-treated controls in how their HRV changed over time. In saline-treated birds, daytime HRV increased over the course of seven days. This shifting to more parasympathetic control suggests that the birds were acclimating to captivity. In phentolamine-treated birds, HRV did not increase, but stayed low throughout the week. Therefore, treating with an alpha-blocker led to higher SNS activation/lower PNS activation over one week. As a consequence, phentolamine treatment possibly resulted in a less favorable outcome than control treatment, although the pattern is not statistically significant at night, when HRV values are probably more reliable.

The continued high sympathetic activity may be explained by the role of alpha receptors in the negative feedback of NE. E and NE are released as hormones from the adrenal medulla. However, they are also released as neurotransmitters throughout the nervous system. Although phentolamine cannot cross the blood-brain barrier, it can act on nerves in the periphery (Langer, 1980). Alpha receptors are involved in an autocrine feedback loop on presynaptic neurons for the regulation of NE both in the brain and in peripheral nerves. During stress, NE is released into the synapse, where it binds to alpha2A receptors on the presynaptic neuron to shut down further NE production (Callado and Stamford, 1999; Langer, 1980). By blocking the alpha receptors with phentolamine, the feedback loop is broken and more NE signaling will occur. This temporary disturbance of NE regulation appears to have long-term consequences. Even long after phentolamine

treatment has stopped, SNS activity remains high. During chronic stress, tree shrews upregulated the alpha2A receptors in their brain (Fluegge *et al*., 2003), increasing the sensitivity of the feedback loop. However, if phentolamine disrupts this regulatory signaling, the upregulation of alpha2A receptors might not occur and high NE concentrations may continue.

4.5.3 Startle response

In a previous study on European starlings, a striking difference between newly captured birds and long term captives was the severe reduction, almost elimination, of the cardiac response to startle (Dickens and Romero, 2009). Even after 10 days of captivity, the birds showed almost no heart rate reaction to a sudden loud noise. A potentially diminished startle response during the transition to captivity has been reported in other animals. Newly-captured bighorn sheep had lower plasma concentrations of E and NE during an acute stressor than sheep raised in captivity (Coburn *et al*., 2010). Captive harbor porpoises had lower plasma E and NE after being netted and sampled than free-living porpoises (Siebert *et al*., 2011). In house sparrows in this study, the startle response was somewhat reduced but not to the same degree as in European starlings. There was no statistically significant difference in integrated heart rate between recent captives and one month captives, and no difference between treatment groups. However, maximum heart rate relative to baseline during a startle response was higher in one month captives compared to recently-captured animals. This may have been due to the higher baseline heart rate in newly-captured birds – the heart may not be able to beat any faster. Propranolol treatment may have affected maximum heart rate –

propranolol-treated birds had a higher maximum heart rate on day 7 than they did on day 1. The loss of the startle response could profoundly impact the well-being of birds. While the detrimental effect of an impaired startle response on survival has not, to our knowledge, been tested directly, a study in wild and domesticated lines of Atlantic salmon showed that a reduced cardiac response to a simulated predator was correlated with reduced escape behaviors (Johnsson *et al.*, 2001). If birds are brought into captivity for translocation, an impaired startle response at release could potentially reduce their ability to escape from predators (see Dickens *et al.*, 2010 for a review on the effects of stress on translocation success). Therefore, retaining a healthy startle response, perhaps by the use of propranolol, could be important for captive birds. An experimental evaluation of the connection between the startle response and survival would be an exciting avenue for future research.

4.5.4 Conclusion

Treatment with propranolol appeared to have a net positive effect by attenuating the development of some chronic stress symptoms in house sparrows during the first week of captivity. Propranolol prevented the increase in baseline CORT caused by captivity. Propranolol treatment also caused an increase in maximum startle response over the first week of captivity, though it was still not as high as one month captives. Phentolamine treatment did not have an effect on any chronic stress symptoms we measured. Neither propranolol treatment nor phentolamine treatment significantly affected heart rate during the first week of captivity, though phentolamine may have an adverse effect in keeping HRV low and PNS activity high.

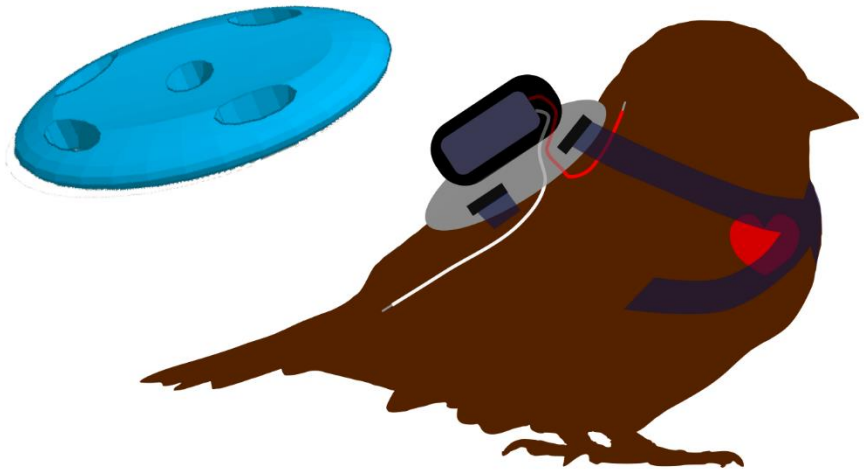

Figure 4.1 Heart rate transmitter harness design. The base of the harness is 3D printed from light plastic. The transmitter is sewn into a small fabric bag and attached to the base. Four straps (5mm satin ribbon) are attached to the holes at the corners of the base and sewn together in the front of the animal's chest. The leads are implanted under the skin and sutured to the muscle at the back of the neck and base of the spine.

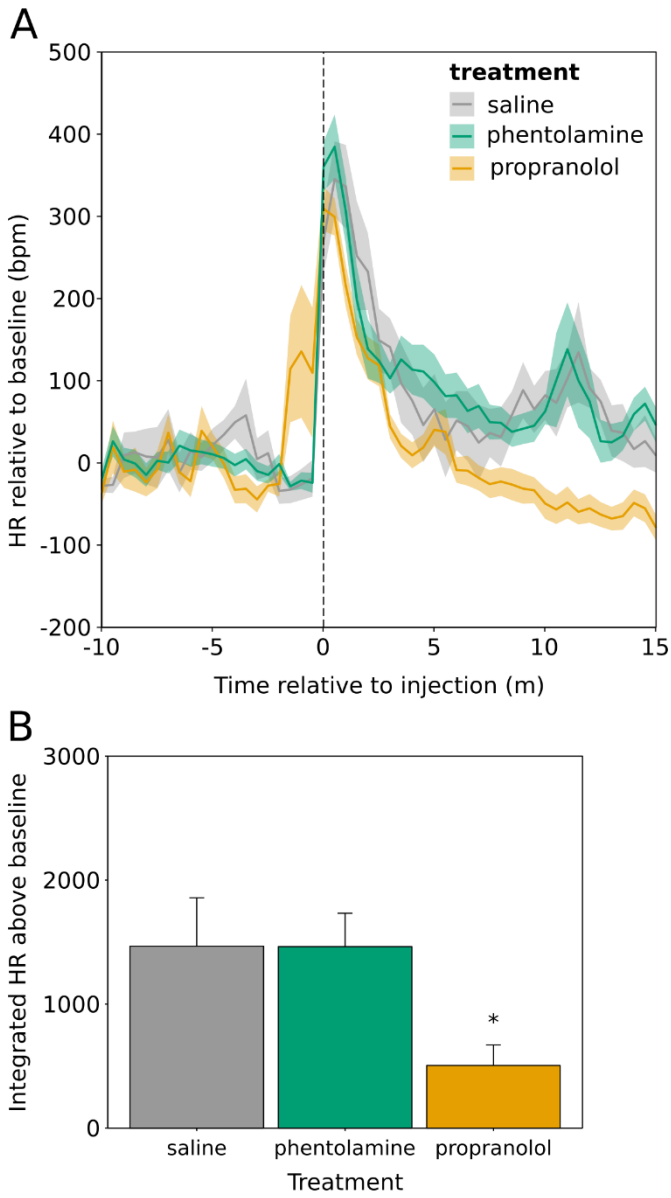

Figure 4.2 Injection of propranolol results in reduced heart rate (HR) relative to saline or phentolamine injection. A) Heart rate relative to baseline. The shaded area indicates one standard error around the mean. Heart rate was recorded before disturbance and after the experimenter had left the room at t=0. B) Integrated heart rate relative to baseline from 0-15 minutes (i.e. area under the curve). Error bars represent mean+SE. *: significant difference ($p<0.05$) relative to saline and phentolamine.

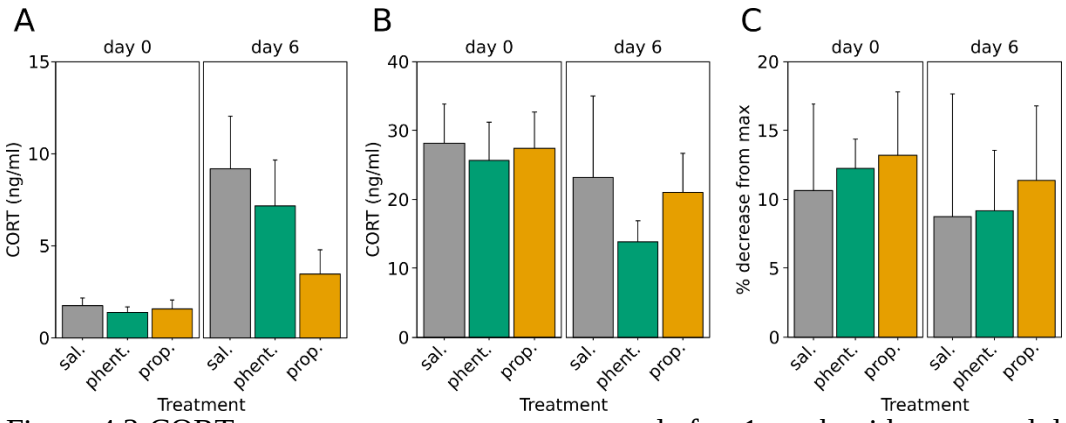

Figure 4.3 CORT response to stress at capture and after 1 week with propranolol and phentolamine injection. A) Baseline corticosterone concentrations taken within 3 minutes of capture or disturbance. B) Stress induced CORT was taken after the birds were held for 30 min in a cloth bag. C) Strength of negative feedback is calculated as the percent decrease in CORT concentration from the stress induced sample 90 minutes after injection with dexamethasone. Error bars represent means+SE.

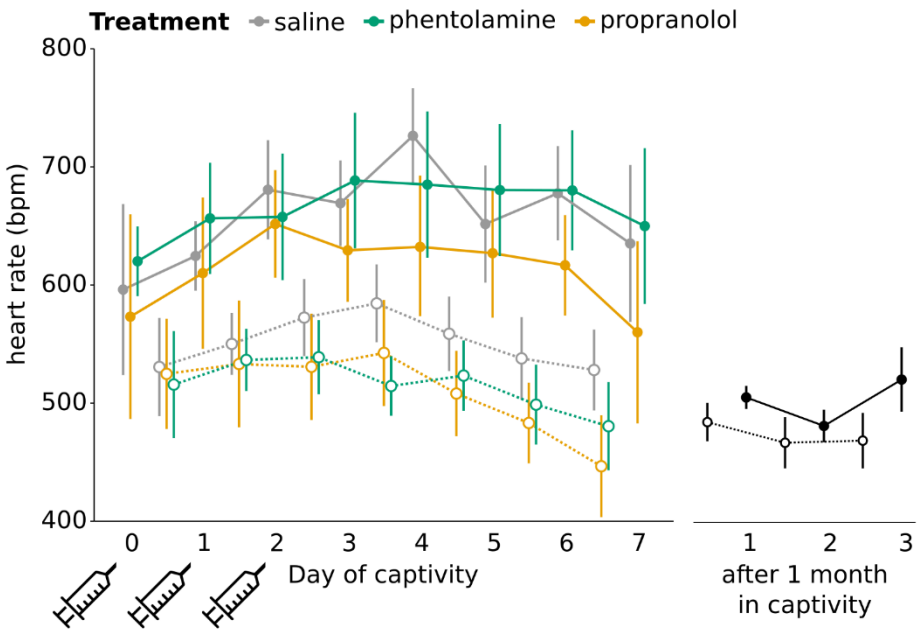

Figure 4.4 Resting heart rate over the course of the first week of captivity with propranolol and phentolamine treatment. Syringes indicate days of saline, propranolol, or phentolamine treatment. Daytime heart rate (solid lines and filled circles) is higher than nighttime heart rate (dotted lines and empty circles) during the first week and after 1 month in captivity. Daytime heart rate is higher in newly captive birds than birds kept in captivity for 1 month. There were no differences between treatment groups. Error bars represent mean±SE.

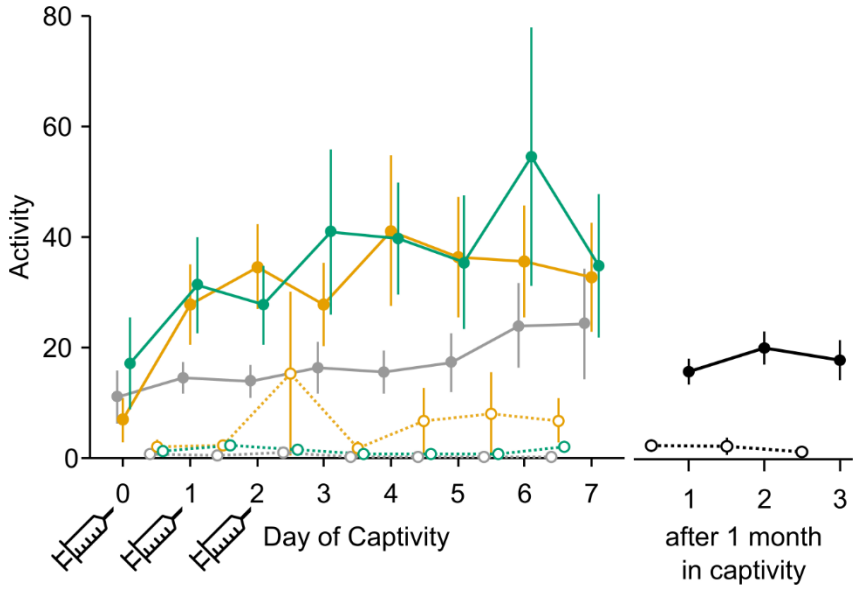

Figure 4.5 Activity levels (a unitless metric) are greater during the day than at night, with no effect of α- or β-blockers. Syringes indicate days of saline, propranolol, or phentolamine treatment. Daytime heart rate (solid lines and filled circles) is higher than nighttime heart rate (dotted lines and empty circles) during the first week and after 1 month in captivity. Daytime heart rate increases over the course of the first week of captivity. Error bars indicate mean+SE.

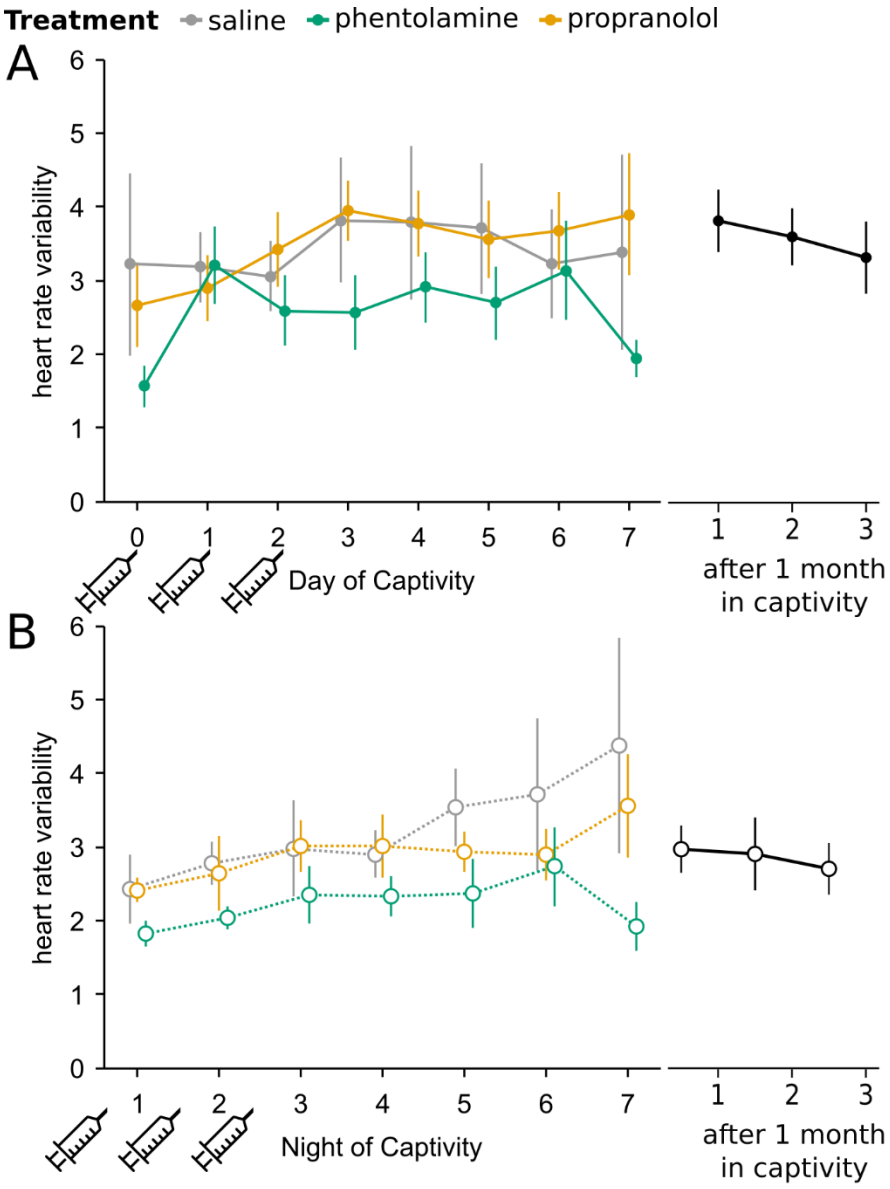

Figure 4.6 Heart rate variability during the first week of captivity with propranolol and phentolamine treatment. HRV is higher during the day than at night in recent captives and after 1 month captivity. A) Daytime HRV increases over the first week of captivity. B) Nighttime HRV increases over the course of the experiment in saline- but not propranolol- or phentolamine-treated birds. Syringes indicate days of saline, propranolol, or phentolamine treatment. Points represent mean±SE.

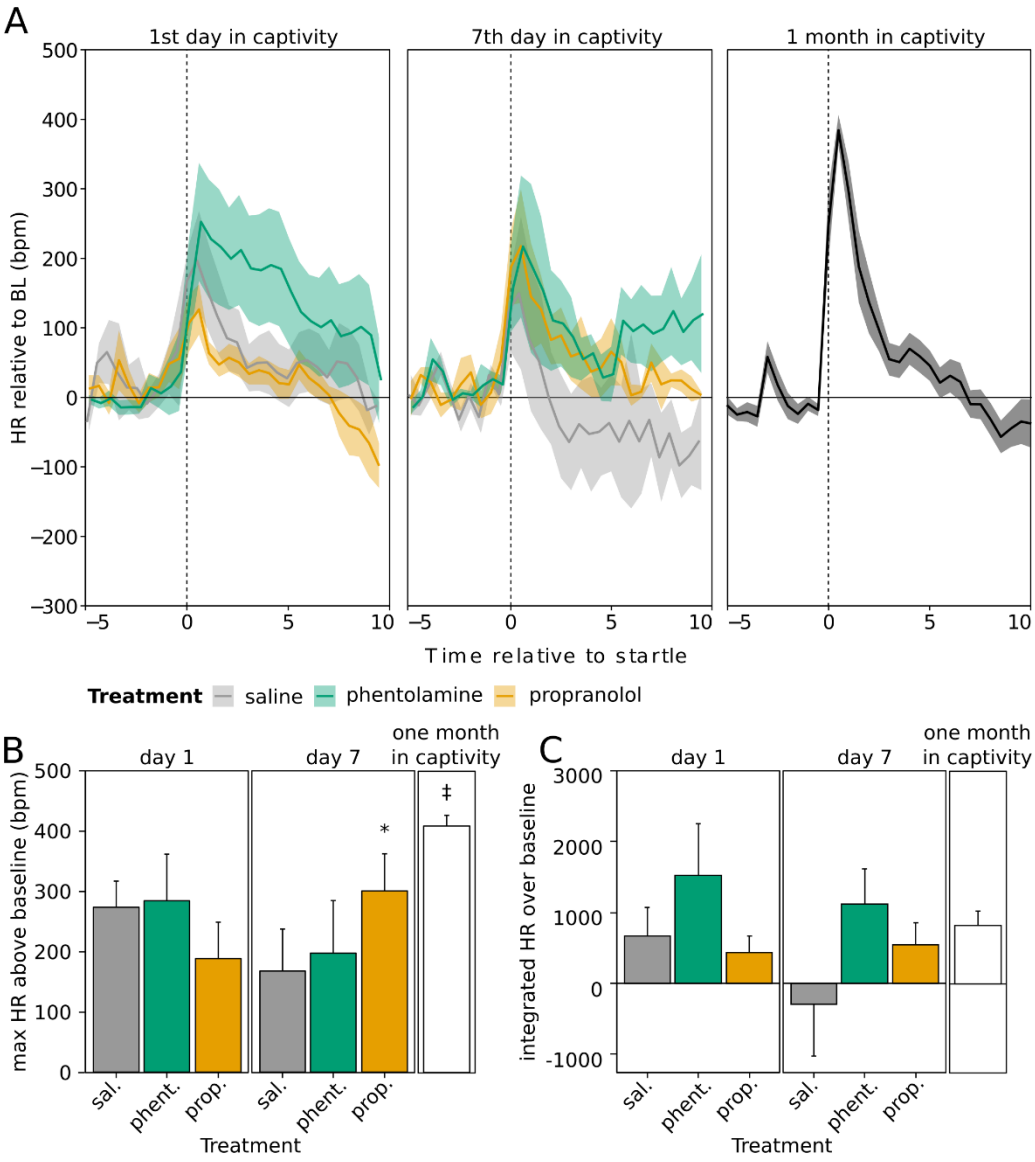

Figure 4.7 Startle tests on the first and seventh day of captivity and after one month of captivity with propranolol and phentolamine treatment. A) Heart rate trace. Shaded area indicates one standard error around the mean. B) Maximum heart rate after the startle. *: $p<0.05$ compared to 1st day of captivity; ‡: $p<0.05$ compared to day 1 and day 7. C) Integrated heart rate.

CHAPTER 5: THE EFFECTS OF DAILY MITOTANE OR DIAZEPAM TREATMENT ON THE FORMATION OF CHRONIC STRESS SYMPTOMS IN NEWLY-CAPTURED WILD HOUSE SPARROWS

Clare Parker Fischer and L. Michael Romero

5.1 Abstract

Wild animals brought into captivity frequently experience chronic stress and 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 to 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. After a week in captivity, baseline corticosterone was higher regardless of treatment group, while stress-induced corticosterone did not significantly increase above baseline in any treatment group. However, mitotane treatment did have some physiological effects, as it reduced resting heart rate and reduced 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 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.

5.2 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 mediate many systems to help direct resources towards immediate 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 in the brain to change the perception of stress, and mitotane acts at the adrenal cortex to reduce the body's ability to mount an HPA response.

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 Morgan and Tromborg, 2007). 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. Captivity can cause glucocorticoid concentrations to change in mammals (Cabezas *et al.*, 2007; Franceschini *et al.*, 2008; Terio *et al*., 2004), birds (Adams *et al*., 2011; Dickens *et al*., 2009b; Fischer and Romero, 2016; Fokidis *et al.*, 2011; Lattin *et al*., 2012a), reptiles (Jones and Bell, 2004), and amphibians (Narayan *et al*., 2011). Results from these studies suggest that chronic captivity stress can be detected by (but is not necessarily limited to) the following symptoms: 1) weight loss, 2) changes in HPA axis function, 3) increase in resting heart rate, 4) increase in resting sympathetic nervous system activity, and 5) abnormal heart rate response to a sudden noise (startle response).

The response of house sparrows to chronic captivity stress has been examined in two previous studies. Newly captive house sparrows had increased baseline corticosterone (CORT, the main glucocorticoid hormone in birds)

compared to free-living birds (Lattin *et al*., 2012a; Fischer and Romero, 2016) and had a higher resting heart rate and a moderately reduced startle response compared to long term captives (Fischer and Romero, 2016). 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 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 a stronger response to startle compared to 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). It also can 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 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 5.3.5), response to startle, and plasma CORT over the course of one week.

5.3 Methods

5.3.1 Drug validations and one month in captivity heart rate

Eight birds were captured in Medford, MA and held in captivity for four weeks. After this period of acclimation, the birds were fitted with heart rate transmitter harnesses (see section 5.3.4). We used these birds to record the following: resting heart rate and HRV; 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 HRV were recorded (see section 5.3.5) for three minutes every two hours for three days while the birds were left undisturbed except for normal animal care. The heart rate response to

startle was recorded by measuring heart rate for 10 minutes, then suddenly opening and closing the door of the bird room and recording 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 three minutes of entering the room and then injecting birds with 0.5mg/kg diazepam or saline. We selected a low dose of 0.5 mg/kg because diazepam at higher doses is a sedative. In zebra finches, a fairly deep sedation is reached at 5 mg/kg (Prather, 2012), so we selected 1 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 two days later but birds who had received diazepam now received saline and vice versa. Plasma CORT was analyzed as below. We then determined the acute effect of diazepam, peanut oil, and mitotane injection on heart rate. 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 one 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 (Dr. Christine Lattin, personal communication).

In a second set of seven birds (also captured in Medford, MA and held in captivity for at least four weeks), we assessed the behavioral 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 the 10 minutes before and 10 minutes after injections.

5.3.2 Experimental design – mitotane and diazepam during the introduction to captivity

A new set of house sparrows were captured in Medford, MA between September 8 and November 6, 2015. 24 animals were used in the final experiments, eight in each treatment group. Immediately at capture a series of blood samples was taken for CORT analysis (see section 5.3.3). The birds were fitted with a harness-mounted heart rate transmitter device within 3 hours of capture (see section 5.3.4). 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 (100μL), mitotane (225 mg/kg body weight in peanut oil), or diazepam (0.5mg/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 one week. Heart rate was automatically sampled for three minutes every two hours. On day 6, another series of blood samples was taken for CORT

analysis. On days 1 and 7 (before their daily injection), the birds' startle response was measured. Heart rate was recorded for 10 minutes before the startle. At t=0, the door to the room was suddenly opened and closed. Heart rate was recorded for a further 10 minutes.

All experiments complied with Association for Assessment of Laboratory Animal Care guidelines and were approved by the Tufts Institutional Animal Care and Use Committee.

5.3.3 Plasma sampling and CORT analysis

On days 0 and 6, a series of blood samples was taken. A baseline sample was collected within three 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). 90 minutes after DEX injection, a final blood sample was collected. For each sample, the alar vein was punctured and ~40µl blood was collected in a heparinized capillary tube. All blood samples were stored on ice and centrifuged at ~1200g 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%.

5.3.4 Heart rate transmitter harnesses

We used the Data Sci International PhysioTel ETA-F10 model of heart rate transmitter. These transmitters measure 19x13x6mm and weigh 1.6 grams. 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 which was secured to a 3D printed base with the leads exposed. Four 3cm lengths of 0.5 cm wide satin ribbon were sewn to the base to serve as straps. Anesthesia was induced with 4.5% and maintained at about 2.5% isoflurane using a vaporizer (Vet Equip, Livermore, CA, USA). At the beginning of surgery, birds were injected intramuscularly with 1mg/kg carprofen as an analgesic. Three surgical sites were sterilized with iodine and alcohol: at the mid-back, near the shoulder, and near the hip. 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 exposed ends of the leads were sewn to the muscle using 4-0 synthetic monofilament suture (Ethicon, Somerville, NJ). All surgical sites were closed with suture and VetBond (3M Animal Care Products, St. Paul, MN, USA). The base of the harness completely hid the transmitter leads that extended out of the skin. The ribbons of the harness were sewn together at the center 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. We monitored the birds until they recovered from the anesthetic. We had an 80% success rate with this procedure – 6 of 30 birds died in surgery or shortly afterwards, leaving a final sample size of 24.

5.3.5 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 birds had recovered from surgery, been given their first injection, and would be left undisturbed for the night) a three-minute sample was recorded every two hours. 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 relative signal strength between the receivers is interpreted as movement within the cage, and is recorded as a unitless value. Activity was analyzed on DataScience's Analysis software.

Heart rate data were analyzed 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.

5.3.5.1 Heart rate variability analysis

HRV 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 ~230

heartbeats for each 3-minute sampling window. The trace was visually inspected to ensure accurate identification of R-waves and individual marks were adjusted as necessary. HRV is a unitless measure adjusted for heart rate, with high HRV indicating beats are more irregular and low HRV indicating that they are more regular. HRV 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 HRV indicates more parasympathetic and less sympathetic activity) (Cyr *et al.*, 2009; Korte *et al.*, 1999; Perini and Veicsteinas, 2003).

5.3.6 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.*, 2015). 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 heart rate, we measured 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 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 analyzed 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 dexamethasone 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 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 HRV, we constructed separate models for daytime and nighttime. We created

linear mixed effects models including treatment, day of captivity, and their interaction, which were analyzed as above. When 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 one month captives is shown in the figures for comparison. However, we did not run statistical models that included these data.

5.4 Results

5.4.1 Validations

The effect of diazepam on the CORT response to stress was analyzed using birds that had been kept in captivity for one month (Figure 5.1A). 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 behavioral effects of diazepam were also analyzed. 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 behavior 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, Figure 5.1B). There were no significant effects of injection of saline or diazepam on any other behavior 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 (Figure 5.1C). 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, Figure 5.1D). A Tukey's post-hoc test found that integrated heart rate after diazepam was higher than after mitotane (z=2.31, p=0.05) and marginally higher than after 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 heart rate to return to within 1 standard deviation of baseline ($F_{2,10.1}$=1.43, p=0.3) between treatments.

5.4.2 Weight loss after one week in captivity

Most birds lost weight over the course of the experiment (29 of 32). There was no effect of 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 one week in captivity.

5.4.3 The hypothalamic-pituitary-adrenal axis

At capture, baseline levels of CORT were below the detection limit of the assay in every sample (Figure 5.2). There was no effect of treatment group on stress-induced CORT at capture 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 one week of captivity, there was no effect of 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 one week of

captivity compared to at capture ($F_{1,23}$=8.93, p=0.007). Stress-induced CORT was not different after one week of captivity compared to 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 to baseline levels ($F_{1,23}$=3.05, p=0.09). There was no significant difference between 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).

5.4.4 Activity

The heart rate transmitters provide a unitless metric of activity (Figure 5.3). We analyzed daytime and nighttime activity separately. 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 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 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 analyzed each treatment group separately to look for an effect of captivity day. There was no effect of day 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).

5.4.5 Heart rate

Heart rate was automatically sampled every two hours. Sampling points where the birds were disturbed were discarded. 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 rate (Fig 4.4: 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 analyzed separately.

For daytime heart rate, we ran a linear mixed effects model including treatment and day of captivity. We found significant effects of 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 one week in captivity. 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).

5.4.6 Heart rate variability

HRV was automatically sampled every two hours. Sampling points where the birds were disturbed were discarded. A repeated measures two-way ANOVA was run including time of day (day or night) and treatment. We found a significant effect of time of day, but no effect of treatment or the interaction between treatment and time of day on HRV (Figure 5.5: 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 rate were then analyzed separately.

Using a two way repeated measures ANOVA, we found no effect of treatment and only a marginally significant effect of day of captivity on daytime HRV (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). HRV tended to increase over the first week of captivity. At night, there was a significant effect of day of captivity but not treatment on HRV (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). Nighttime HRV increased over the first week.

5.4.7 Startle response

The heart rate response to a sudden startle (quickly opening and shutting the bird room door) was recorded on day 1 and day 7 of captivity (Figure 5.6A). Some traces did not have sufficiently distinguishable R waves and were removed from analysis. 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 elevated heart rate.

We analyzed the integrated heart rate on day 1 and day 7 separately to look for an effect of treatment (Figure 5.6B). We found no 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 analyzed each treatment group separately to look for a change in startle response between day 1 and day 7. In the oil and diazepam groups, there was no change in 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, 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 maximum heart rate (within 60 seconds of startle) on day 1 and day 7 separately to look for an effect of treatment (Figure 5.6C). We found no 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 analyzed each treatment group separately to look for a change in maximum heart rate between day 1 and day 7. In the oil, diazepam, and mitotane groups, there was no change in 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 analyzed the duration of the startle response, defined by the time to return to within one standard deviation of baseline heart rate (Figure 5.6D). We found no treatment effect on day 1 or on day 7 (respectively $F_{2,20}$=0.77, p=0.5; $F_{2,15}$=0.91, p=0.4). We also analyzed each treatment group separately to look for a change in startle response duration between day 1 and day 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 to day 1 ($F_{1,5.3}$=7.71, p=0.04).

5.5 Discussion

Captivity can act as a powerful chronic stressor for wild animals. 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. Integrated heart rate after injection was higher after diazepam injection than mitotane injection, and marginally higher than the controls (Figure 5.1D). 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 heart rate (Uzun *et al*., 2006).

We also tested the acute effects of diazepam on CORT and on behavior. 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 to saline injection. Similarly, in wild snow buntings (*Plectrophenax nivalis*), diazepam had no effect on stress-induced CORT in response to capture 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 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 behavior – 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 (Figure 5.2A). This is consistent with previously reported patterns over the first week of captivity in untreated or saline treated house sparrows (Fischer and Romero, 2016; Lattin *et al*., 2012a). Over the course of the first week, our oil-treated birds showed increasing daytime and nighttime activity, daytime heart rate, and HRV 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 a previous study (Fischer

and Romero, 2016). 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 (Dr. Christine Lattin, personal communication). 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 chronic stress protocol (Cyr *et al*., 2009); the increase in heart rate may therefore indicate that our control animals were chronically stressed. In addition, oil itself may also impact 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 (Vanvliet *et al*., 1995). High lipid diets can cause changes in heart rate over the short term as well; rabbits had increased heart rate during the first three days of a high fat diet (Barzel *et al*., 2014). Somewhat paradoxically, however, we also saw increased HRV over the course of the first week, as we had initially predicted. The increase in HRV indicates that the birds were shifting to more parasympathetic control of heart rate, which indicates a reduction in chronic stress. Daytime HRV measurements are probably unreliable, however, as HRV's interpretation relies on a resting animal. If the animal is moving around the cage during the measurement period, 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 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 HRV 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. 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 and colleagues (Lattin *et al*., 2012b). Baseline CORT production after mitotane may be somewhat variable, as when Breuner *et al*. used a higher dose of mitotane, there was no difference from controls in baseline CORT (2000). In our experiment, there was no difference in baseline or stress-induced CORT between mitotane and oil treated birds (Figure 5.2A). However, stress-induced CORT in the oil treated birds was low (11.9±6.1 ng/mL compared to 15-30 ng/mL in untreated or saline treated birds held in captivity for 5-7 days [Fischer and Romero, 2016; Lattin *et al*., 2012a]). 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 to controls that we expected to see, we did see changes in heart rate that are consistent with reduced CORT. Daytime heart rate was lower in mitotane- than oil-treated birds (Figure 5.4). CORT has permissive effects on the action of epinephrine and norepinephrine (Sapolsky *et al.*, 2000). Therefore, a reduction in 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 one-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 impacted by mitotane treatment; heart rate returned to baseline more quickly after a startle in mitotane treated birds (Figure 5.6D). 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 (Figure 5.3); 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 HRV was higher in mitotane treated birds than in oil treated controls (Figure 5.5A). This could indicate higher parasympathetic and less sympathetic nervous system activity. However, as discussed above, daytime HRV 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 HRV 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.

Diazepam, on the other hand, 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 (Abreu *et al*., 2014; Le Fur *et al*., 1979; Roy-Byrne *et al*., 1988). It has also been shown to reduce 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 this 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 behavior (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, 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. However, it should be kept in mind that mitotane's effects can be deleterious and can result in mortality (Breuner *et al*., 2000). Diazepam, on the other hand, resulted in no difference in heart rate, HRV, CORT, or activity. It does not appear to be an effective tool for reducing chronic stress in newly caught wild birds.

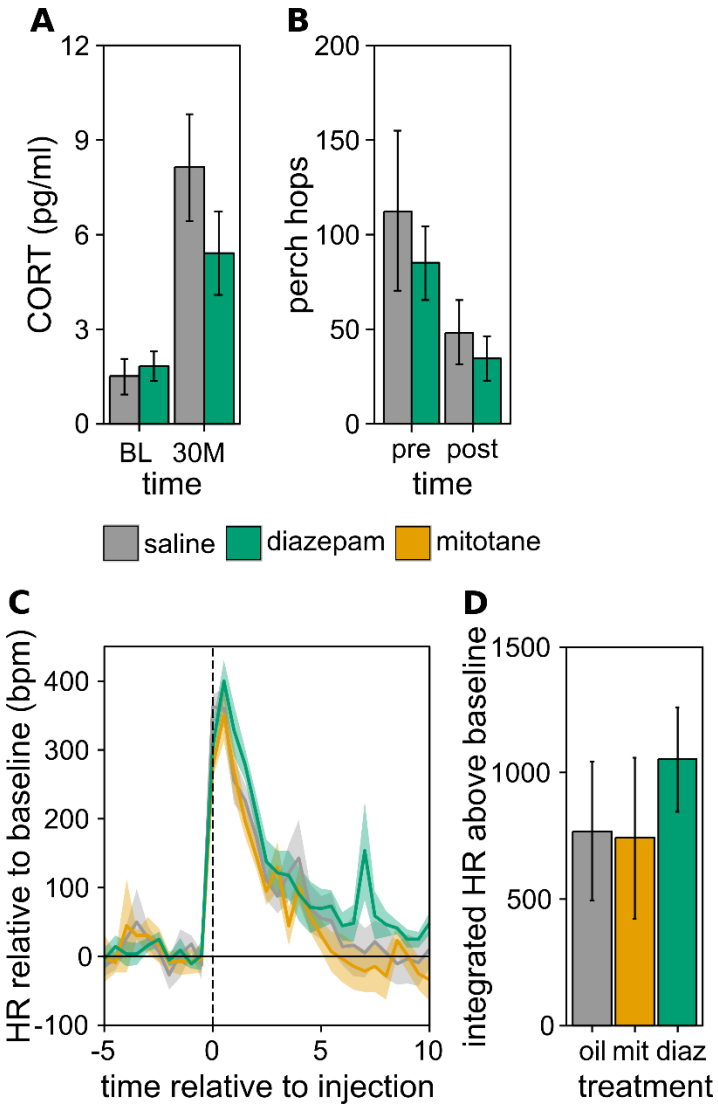

Figure 5.1 Validations of mitotane and diazepam. A) Effect of diazepam on CORT response to stress. 0.5mg/kg diazepam or saline was injected after the baseline blood sample, then birds were held in a cloth bag for 30 minutes. B) Behavioral response to diazepam injection. C) Heart rate response to oil, mitotane, or diazepam injection. The line and shaded areas indicate mean ± standard error. D) Integrated heart rate for the 10 minutes post injection. In A, B, and D, error bars represent mean ± standard error.

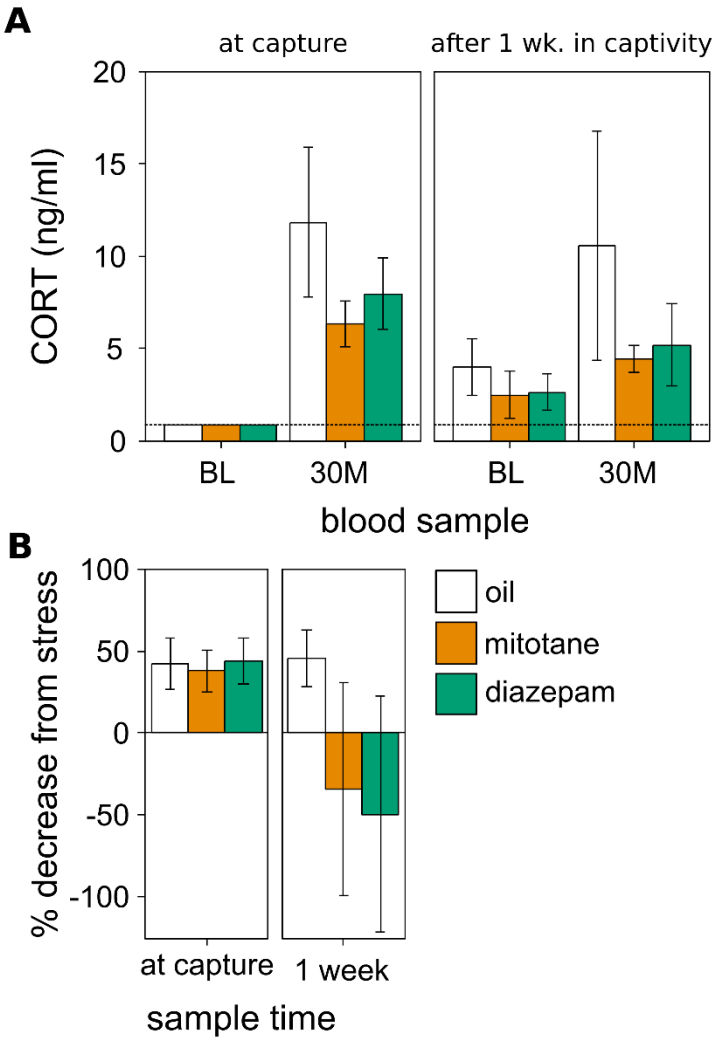

Figure 5.2 HPA response at capture and after one week of captivity with diazepam and mitotane treatment. At capture, plasma was sampled at <3 minutes, birds were held in cloth bags for 30 minutes, and plasma was sampled again. Note that at-capture samples are presented by their ultimate treatment group (oil, mitotane, diazepam), but no treatment was yet applied. They were injected with dexamethasone and held a further 90 minutes to measure negative feedback strength. Birds were held one week and treated with oil (a vehicle control), mitotane, or diazepam daily, and a second stress series was taken. A) Baseline (BL)

and stress-induced (30M) 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 dexamethasone/CORT at 30M)). 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.

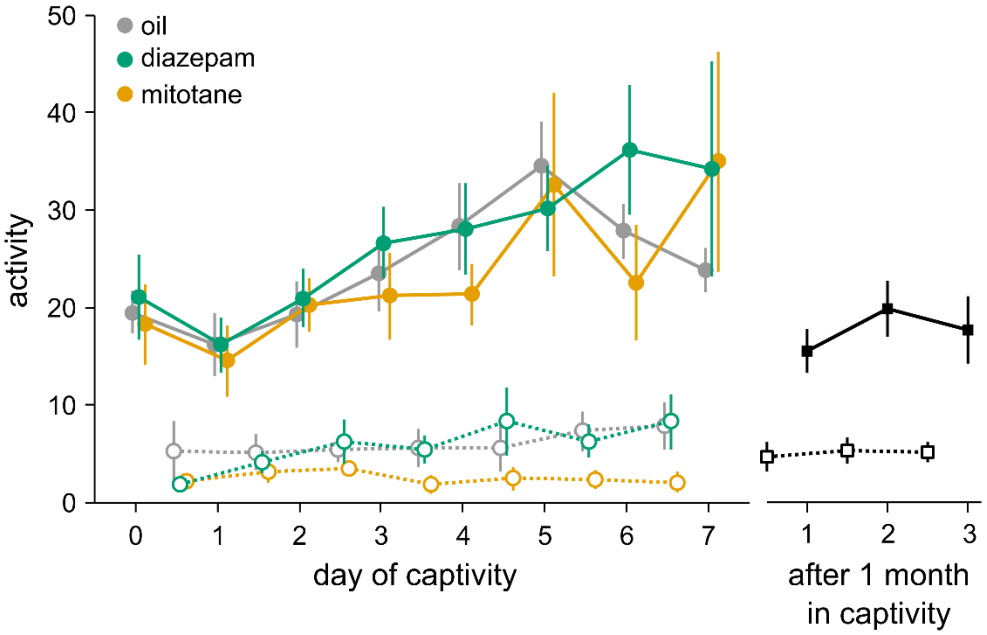

Figure 5.3 Activity during the first week of captivity with diazepam and mitotane treatment. Activity is recorded as a unitless metric from the heart rate transmitters. Birds were fitted with transmitters at captivity and treated with oil, diazepam, or mitotane once daily for the first week of captivity. Filled symbols indicate activity during the lights on period; open symbols indicate activity during the dark period. N=8 for each treatment group. Activities from birds held for one month are included for visual comparison. Error bars represent mean ± standard error.

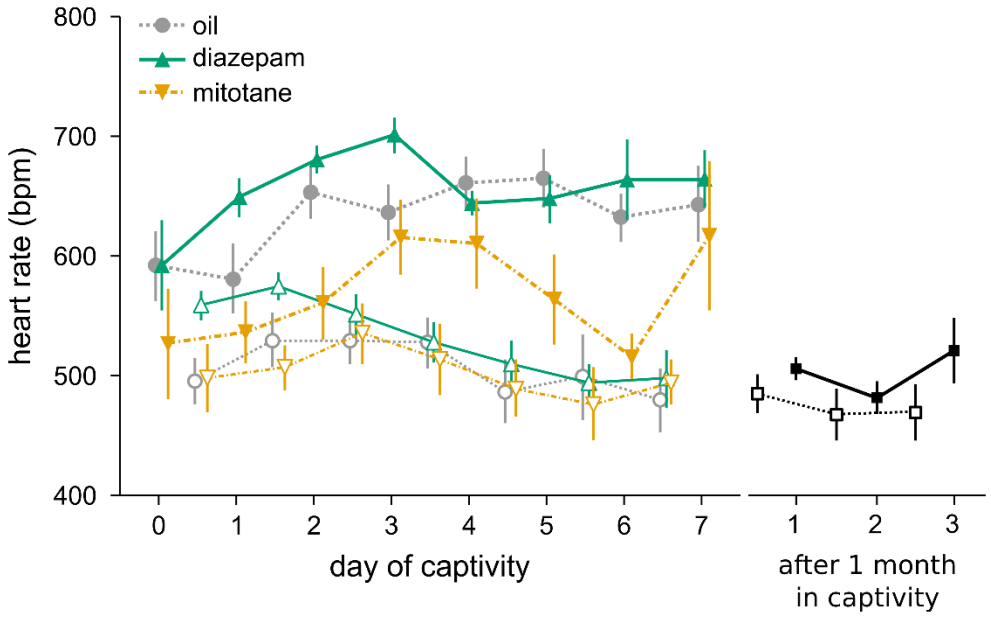

Figure 5.4 Heart rate during the first week of captivity with diazepam and mitotane treatment. Birds were fitted with transmitters at captivity and treated with oil, diazepam, or mitotane once daily for the first week of captivity. Filled symbols indicate activity during the lights on period; open symbols indicate activity during the dark period. N=8 for each treatment group. Heart rates from birds held in captivity for one month and recorded over three days are included for visual comparison. Error bars represent mean ± standard error.

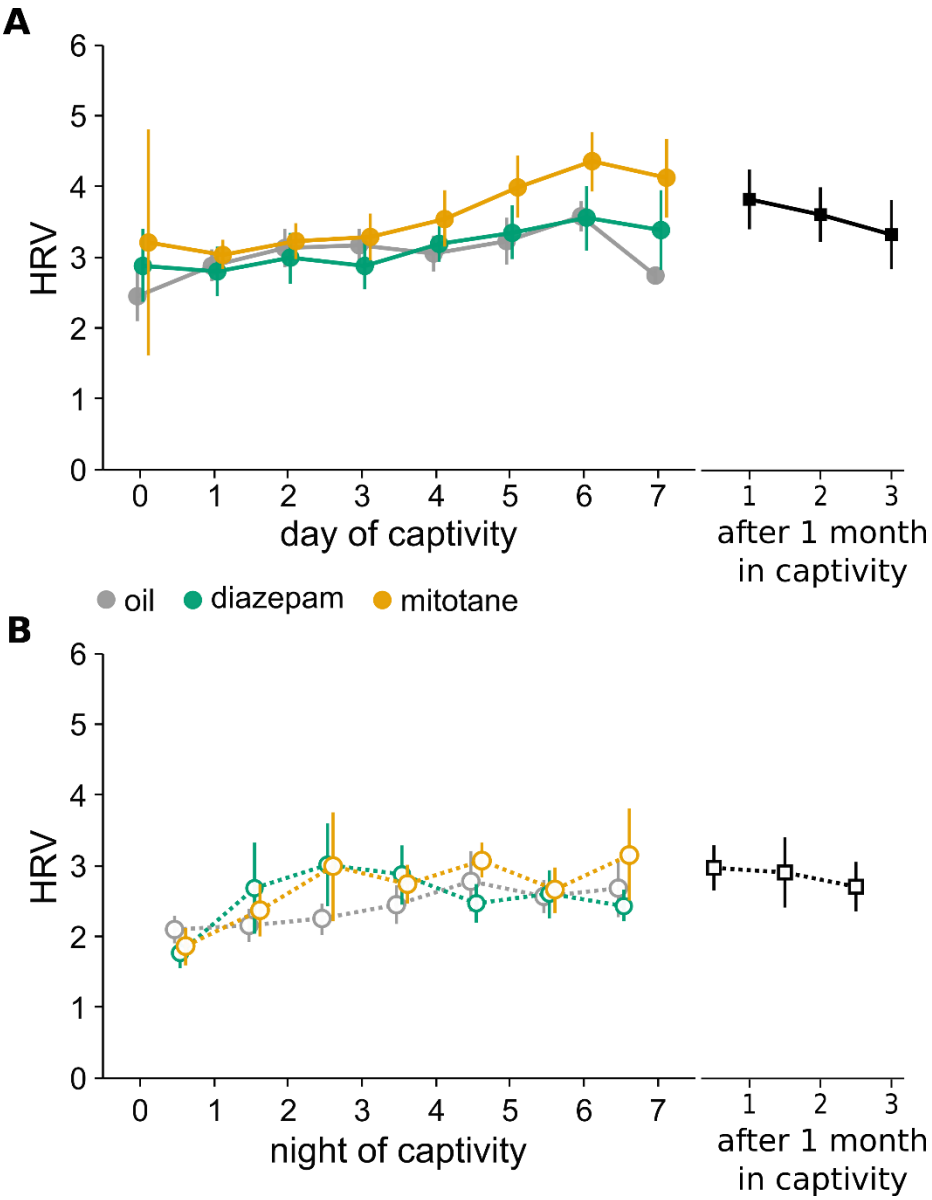

Figure 5.5 Heart rate variability during the first week of captivity with diazepam and mitotane treatment. Birds were fitted with transmitters at captivity and treated with saline, oil, diazepam, or mitotane once daily for the first week of captivity. A) HRV during the light period. B) HRV during the dark period. N=8 for each treatment group. HRV from birds held captive for one month are included for visual comparison. Error bars represent mean ± standard error.

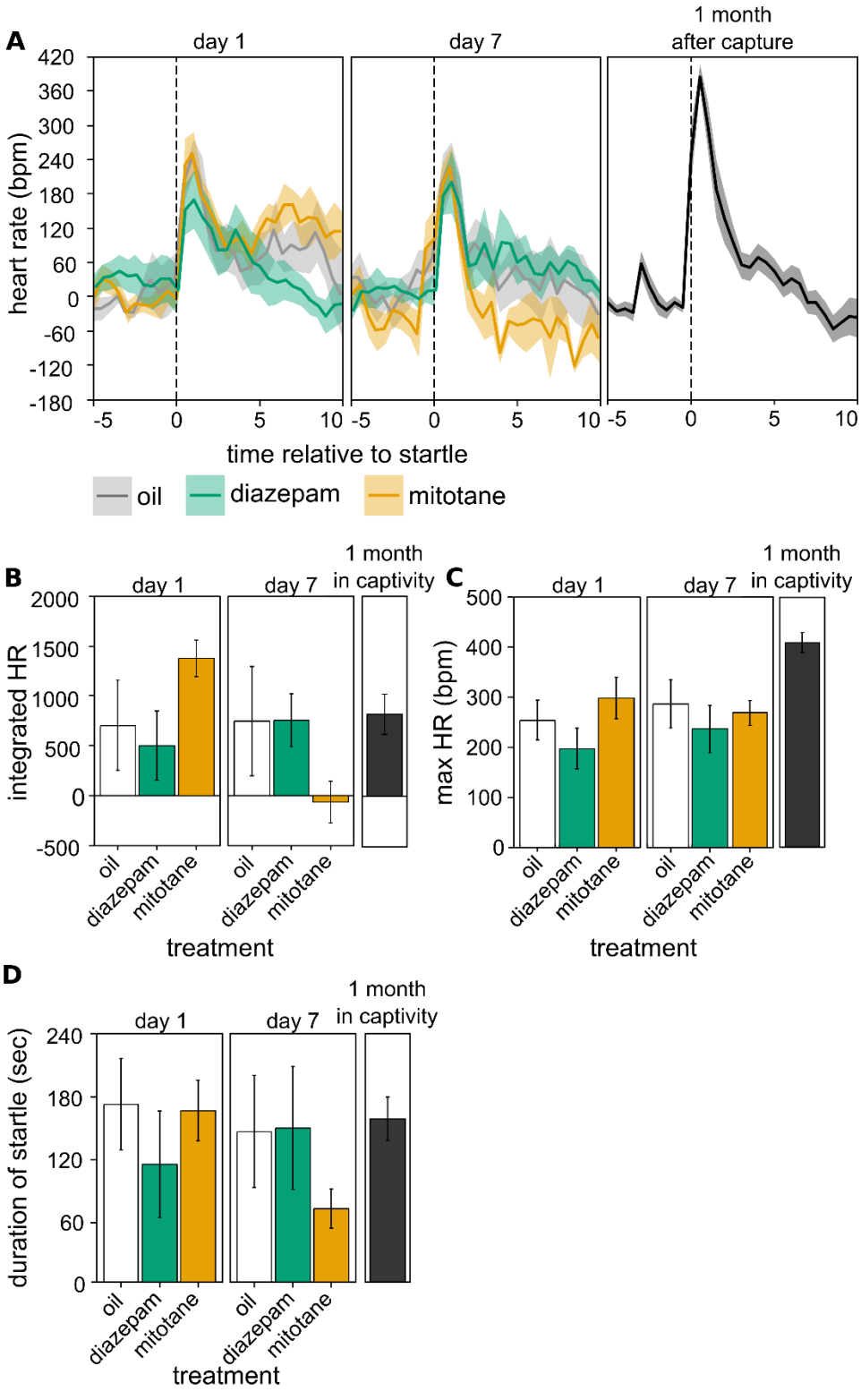
A
day 1
day 7
1 month
after capture
heart rate (bpm)
420
360
300
240
180
120
60
0
-60
-120
-180
-5
0
5
10
time relative to startle
oil
diazepam
mitotane
B
day 1
day 7
1 month
in captivity
integrated HR
2000
1500
1000
500
0
-500
oil
diazepam
mitotane
treatment
C
day 1
day 7
1 month
in captivity
max HR (bpm)
500
400
300
200
100
0
oil
diazepam
mitotane
treatment
D
day 1
day 7
1 month
in captivity
duration of startle (sec)
240
180
120
60
0
oil
diazepam
mitotane
treatment

Figure 5.6 Heart rate response to sudden noise with diazepam and mitotane treatment. 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 one week and treated with oil (a vehicle control), mitotane, or diazepam daily, and a second startle response was recorded. A) Heart rate trace over time relative to 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) 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 baseline heart rate. Error bars indicate mean ± standard error.

CHAPTER 6: SEASONALITY OF NEOPHOBIA IN CAPTIVE HOUSE SPARROWS

Clare Parker Fischer, Devin Merullo, Brenna M. Gormally, and L. Michael Romero

6.1 Introduction

Neophobia, the "fear of the new," is a behavioral trait displayed by many wild animals. When an animal encounters a new object in its environment, it must balance the potential risks involved with the possibility of reward. A new object may be associated with an unknown danger, such as a new predator, or it may offer new resources, such as food or a potential nesting site. Different species and different individuals may weigh these risks and rewards differently, and behave more or less fearfully around novelty (reviewed in Greenberg and Mettke-Hofmann, 2001). For researchers investigating animal personalities, time to explore a novel object or situation is one of the behaviors that is frequently measured to distinguish different temperaments (Réale *et al.*, 2007).

We initially characterized neophobia in captive wild house sparrows (*Passer domesticus*) as a potential behavioral assay that may correlate with glucocorticoid titers. We conducted laboratory-based food-motivated neophobia trials in house sparrows caught in Medford, MA in October 2012. Similar tests of neophobia have been successfully conducted in a number of bird species in captivity (Apfelbeck and Raess, 2008; Boogert *et al.*, 2006; Greenberg, 1992; Martin and Fitzgerald, 2005). The birds' food dishes were removed overnight to presumably create an equal level of hunger, and thus motivation to feed, in all birds.

Food dishes were then replaced in the morning either with or without a novel object and we recorded the latency to approach and feed from the dish. In the initial experiment, most birds showed a clear neophobic response – approaching and feeding from the unmodified dish much sooner than from a dish with a novel object. We repeated this procedure in July 2013, and to our surprise, found that the birds approached the dish much more quickly than the October birds, feeding from both modified and unmodified dishes within seconds.

The primary difference between the trials was that one was conducted in October and the other in July. Was this drastic change in behavior due to seasonal effects? Nearly every temperate species changes its behavior as the seasons change. Furthermore, glucocorticoids, which can have a pronounced impact on neophobia in birds (Lendvai *et al.*, 2011; Schoech *et al.*, 2009), has a strong seasonal pattern in house sparrows (Romero *et al.*, 2006). Based on these observations, we hypothesized that neophobia would be low in house sparrows captured during the breeding season and higher in other times of year, perhaps because of greater food demands during breeding. To test this hypothesis, we repeated our neophobia experiments at different times of year between 2012 and 2016, collecting data in October (near the end of prebasic molt), November (after the completion of molt), March (pre-breeding), April (breeding season), and during two years in July (late breeding/chick rearing) (Anderson, 2006; Lowther and Cink, 2006).

6.2 Methods

At each timepoint (October 2012, July 2013, November 2013, March 2014, April 2016, and July 2016), we collected 7-8 wild house sparrows (22 in July 2016)

from on or around the Tufts University campus (Medford, MA), an urban/suburban area. Birds were brought into an indoor animal facility and individually housed. The photoperiod was set to reflect the light conditions at the time of capture. Seed was provided *ad libitum.* Birds were given two weeks to acclimate to captive conditions.

Neophobia trials were conducted in two steps. The night before a trial, food dishes were removed, scattered seed was removed from the bottom of the cages, and black dividers were placed between the cages to prevent birds from viewing the behavior of their neighbors. In the morning, the food dish was replaced and the birds filmed for 20 minutes, after which the cameras were switched off and the black dividers were removed. This procedure was repeated at least three times on three consecutive days to allow the birds to become accustomed to the procedure. We then conducted behavior trials. On the first trial, birds would be given their normal food dish (control) for the first day. On the following day, their food dish would be returned with a novel object placed on, near, or around the dish. The novel objects are listed in Table 2 and were chosen at random for each trial. These two trials (control followed by novel object) were repeated two more times with different objects, so each bird was recorded approaching 3 different novel objects. There were no differences between the different objects in eliciting neophobia (data not shown). Because we were interested in the effect of novelty itself and not the effects of each object, we coded all novel objects as "with object" in our statistical models. In July 2016, the animals were part of another experiment and were only recorded for one control (no object) and one novel object trial. For each video, we

scored the amount of time (i.e. latency) for the birds to approach (perch on or right next to the dish for at least 1 second) and feed from their dishes. Timers were started when the door to the bird room closed behind the researcher. Birds that approached or fed before the door closed were assigned a minimum score of 0 seconds for analysis; birds that did not approach or feed within 20 minutes were assigned a maximum score of 1200 seconds.

All statistical analyses were conducted in R (Version 3.1.3; R Core Team, 2013). The design for our analyses was unbalanced because we had different numbers of control and novel object trials at the different timepoints and different numbers of birds per timepoint. Furthermore, our data were non-normal. Therefore, we constructed generalized linear mixed effects models using the "glmer" function in the lme4 package of R (Bates *et al.*, 2015) with individual bird as a random variable and a lognormal distribution. We compared our models to a null model (with no fixed effects) using a Likelihood Ratio Test. We followed this with a Tukey's multiple comparison test if warranted using the glht function in the "multcomp" package of R (Hothorn *et al.*, 2008). In the figures, different letters indicate significant differences between timepoints. This method was used for all analyses. All data are presented as mean ± standard error.

6.3 Results

We analyzed the six different repetitions of the experiment as separate seasons – (March 2014, April 2016, July 2013, July 2016, October 2012, and November 2013). We compared the latency to approach the unmodified food dishes (controls) across seasons (Figure 6.1A, white bars), and found that the model

including season was significantly better than the null model (χ^2=75.27, df=5, p<0.0001). A Tukey's multiple comparison test found that birds approached the food dish more quickly in July 2013 than in any other month, and they approached more quickly in October than in March, April, July 2016, or November (p<0.001 for all). There was also a seasonal effect of latency to approach a food dish with a novel object (Figure 6.1A, black bars; χ^2=64.70, df=5, p<0.0001). Birds approached novel objects significantly more quickly in July 2013 than in any other month (p<0.001 for all).

We then tested whether novel objects caused a difference in approach time within each season (comparing latencies to controls and novel objects within the same season). Approach latency was significantly longer with a novel object in all seasons (March: χ^2=28.81, df=1, p<0.0001: April: χ^2=12.31, df=1, p=0.0004; July 2013; χ^2=6.61, df=1, p=0.01; July 2016; χ^2=84.34, df=1, p<0.0001; October: χ^2=24.38, df=1, p<0.0001; November: χ^2=8.37, df=1, p=0.004). However, the magnitude of the response was quite variable, and mean approach time to novel objects was less than a second in July 2013 (i.e. most birds approached before the researcher left the room, and all approached within 5 seconds).

In July 2013, we may have recorded extreme and unrepresentative behavior (see Discussion). We therefore reran the same statistics after removing the July 2013 trials. With control dishes, the model that included season was significantly better at explaining the variance in the approach latency times than the null model (χ^2=22.06, df=4, p=0.0002). Birds approached more quickly in October than in every other season (p<0.001 for all). There was no seasonal effect on approach

latency in trials with a novel object when July 2013 was removed (χ^2=3.96, df=4, p=0.4).

We repeated the same analyses for feed latency instead of approach latency, and found very similar patterns (Figure 6.1B). For control trials, there was a significant effect of season on feed time (Figure 6.1B white bars; χ^2=108.0, df=5, p<0.0001). Birds in July 2013 fed more quickly than in every other month (p<0.001 for all). Birds in July 2016 and October fed more quickly than in March, April, or November (p≤0.05 for all). There was a seasonal effect on feed latency with novel objects present as well (Figure 6.1B black bars; χ^2=94.27, df=5, p<0.0001), with faster feed latency in July 2013 than in every other season (p<0.001 for all).

The presence of a novel object increased latency to feed in all six seasons tested (comparing latencies to controls and novel objects within the same season). (March: χ^2=27.10, df=1, p<0.0001; April: χ^2=15.67, df=1, p<0.0001; July 2013: χ^2=4.82, df=1, p=0.03; July 2016: χ^2=90.06, df=1, p<0.0001; October: χ^2=13.39, df=1, p=0.0003; November: χ^2=12.91, df=1, p=0.0003).

If July 2013 was removed from the feed latency statistics, the model containing season explained the feed latency data for the control trials better than the null model (χ^2=25.75, df=4, p<0.0001). Birds in July and October fed more quickly than in March, April, or November (p<0.05 for all). However, there was no effect on feed times when a novel object was present (χ^2=6.07, df=4, p=0.2).

There was a great deal of individual variability in approach and feed latencies. Individual approach latencies with and without a novel object are shown

in Figure 6.2. Latency to feed showed a similar degree of individual variability (data not shown).

6.4 Discussion

Researchers have long used neophobia as an assay of animal behavior. In a frequently-used experimental design, animals are motivated by hunger to approach a food source where a novel object has been placed (Apfelbeck and Raess, 2008; Boogert *et al.*, 2006; Greenberg, 1992; Martin and Fitzgerald, 2005). Because many behaviors are seasonal, neophobia may be different if measured at different times of year. House sparrows have clearly defined breeding, molting, and wintering seasons with different energy requirements and behaviors in each (Anderson, 2006). We found seasonal differences in motivation to feed in captive house sparrows. When subjected to overnight food deprivation and presented with an unmodified food dish, birds tended to approach and feed more quickly in July and October than in March, April, and November. July is late in the breeding season for house sparrows, an energy-intensive time. However, as all our birds were held in single cages in captivity, none of them currently had offspring to provision. The annual molt falls between August and October in house sparrows, meaning our October sample was likely near the end of molt (although molt status was not recorded). This is another energy-intensive season; birds must shed and regrow their feathers, which total up to 7% of their body mass in this species (Schifferli, 1981 as cited in Anderson, 2006).

In July 2013, we saw a very attenuated neophobic response, with most birds approaching the modified and unmodified food dishes before the researcher had

left the room. While we saw a slight neophobic response in feed latency, it lasted on average only 11±4 seconds. In this case, it appears that the motivation to feed was so strong it overwhelmed any fearfulness. The amount of time the animals were deprived of food was similar at every month, about 12-15 hours. This corresponds to the length of time of the dark period during winter; as house sparrows do not forage at night, they are able to tolerate a period of this duration without food. In July 2013, the birds had a long light cycle (15 hours light, 9 hours dark), which was set to correspond with the times of sunrise and sunset in Massachusetts in July. In order to reduce the differences in the night-time fasting period between summer and winter, we conducted the neophobia trials on average 2.5 hours after lights-on during this month. If birds tend to forage in the morning, they may have been particularly hungry even with the same length of food deprivation. In July 2016, the birds were exposed to the same duration of light and darkness, but trials were conducted approximately 1 hour after lights-on. This difference could potentially explain the different responses in July. It is not clear whether the July 2013 data (with a longer morning without food but comparable food restriction to other seasons) or the July 2016 data (with shorter overall food restriction period but comparable morning period without food) are best when comparing to the other seasons. However, if we remove the July 2013 data from our analyses, there were still seasonal differences in approach and feed latencies in control trials, though the seasonal effect on approach and feed times with a novel object disappeared. This indicates that the overall conclusions of seasonal differences in neophobia do not solely reflect the exceptional data collected in July 2013. In support, birds

continued to have a stronger motivation to feed during October and July than November. We hypothesize that motivation to feed is increased during times of high energy demand (chick rearing and molt).

These results raise concerns about designing behavioral assays that may be compared between different seasons. If food is removed overnight, is it more important that the total number of hours the food has been removed is kept constant? Or is it more important that the food be removed at the same time relative to lights-out and replaced at the same time relative to lights-on? However, the striking and significant difference between approach latency with no object controls in October (26±10 seconds) and November (519±99 seconds) suggests that underlying motivational differences exist that are unrelated to day length. The difference in day length between these two periods was only 1 hour, so the duration of time when they would have naturally been unable to feed in the wild was approximately the same.

In addition to potential seasonal differences in motivation to feed and neophobia, we also found a great deal of individual variability in behavior (see Figure 6.2). For example, in March, the individual on the far left of the graph approached before the researcher had left the room for all three no object trials and all three novel object trials, showing a high motivation to feed and no apparent neophobia. In the same month, the individual on the far right did not approach the dish during the 20-minute observation period for any trial except the first control trial. In this case, it was impossible to tell whether the animal displayed neophobia or not. In European starlings (*Sturnus vulgaris*), a similar behavior was seen, where

exposure to a novel object once affected behavior to later no-object trials (Fischer *et al.,* 2016). Duration of latency to approach or feed from a control dish did not appear to be related to whether the animal showed neophobia – not all animals showed a difference between control and novel-object trials regardless of their control latency. Neophobia is often used as part of the assessment of animal personalities. Personalities are defined as a suite of behavioral traits that are constant throughout an animal's life and in different circumstances (Réale *et al*., 2007). If a low neophobia bird was tested in October and again in March, the theory of animal personalities predicts that it would continue to have low neophobia relative to the population, even if the behavior seasonally shifted. As we tested different animals in every timepoint, we are not able to address the question of whether neophobia is consistent in individual house sparrows.

Overall, we found evidence that motivation to feed is a trait with underlying seasonal variation. Although we do not have strong evidence that approach latency to a novel object seasonally fluctuates (given that the seasonal trend is driven by the extreme values in July 2013), these results are still relevant for researchers who use neophobia as a behavioral assay. If a neophobia score is assigned based on difference from control (novel object latency minus control latency), then seasonal effects on food motivation can impact neophobia score. Neophobia scores may appear to increase during times of high food motivation if control latencies decrease. However, these potential seasonal fluctuations may not be relevant to the way an animal perceives novelty. Assessment of the risk a novel object represents likely does not begin only after an animal would have approached an unmodified

food dish, but presumably begins the moment the object enters the animal's field of view. Therefore, potential seasonal effects on motivation should be considered as possible explanatory variables, and neophobia is probably best compared between individuals within a season.

Table 6.1 Novel objects used to stimulate neophobia.

Red dish	Normal food dish painted red
Red ring	Springy red keychain wrapped around the dish
Red ribbon	Red ribbon strung across the top of the dish
Red light	Flashing red light clipped to the cage so it faces a bird perching on the dish
Yellow keychain	Yellow bottle opener keychain clipped to the cage behind the dish
Colored egg	Colorful plastic egg placed in the food dish
Cover	White cover placed over the food dish with an opening to feed through

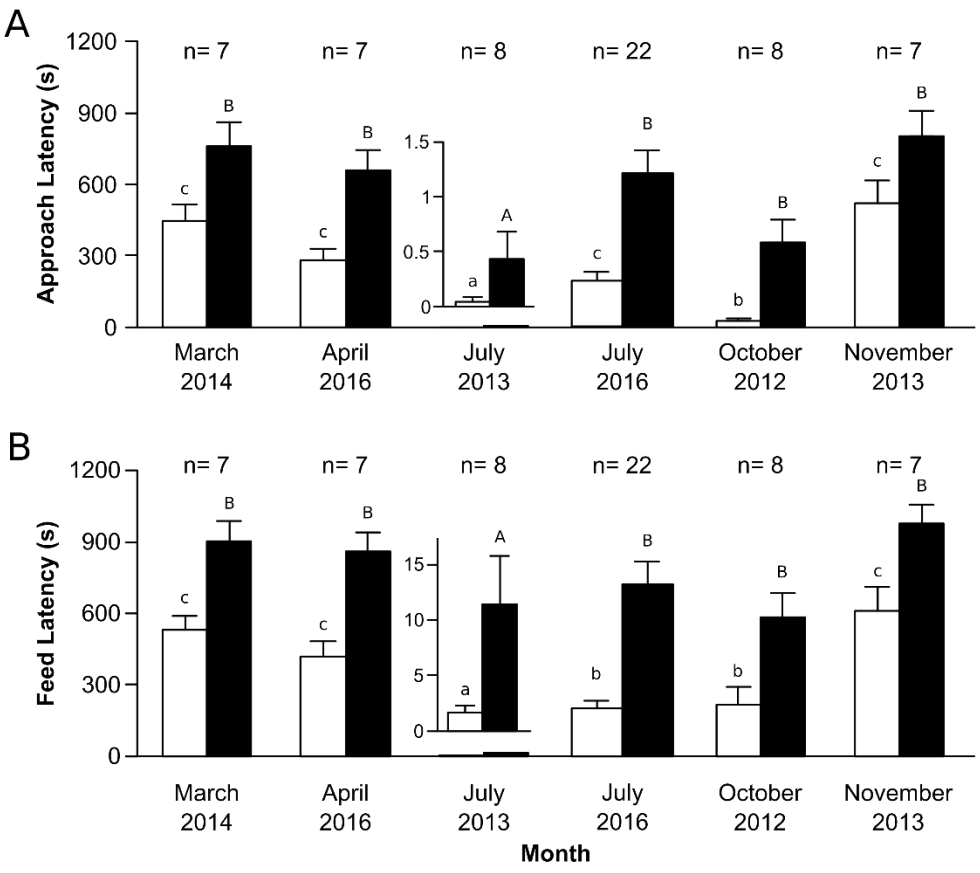

Figure 6.1: Seasonal effects of neophobia in house sparrows. A) Time to approach the food dish varies by season both without novel objects (white bars) and with novel objects (black bars). Latency to approach with a novel object is significantly longer than control latency in all months ($p<0.05$). B) Time to feed varies by season with no object and with novel objects. Different letters indicate differences between months (lower case letters are without objects, capital letters are with novel objects). Latency to feed with a novel object was significantly longer than control latency in all months ($p<0.05$). July 2013 had very low approach and feed times. Inserts are expanded July 2013 numbers – note that the scale changes (units are still seconds). Error bars indicate mean ± standard error.

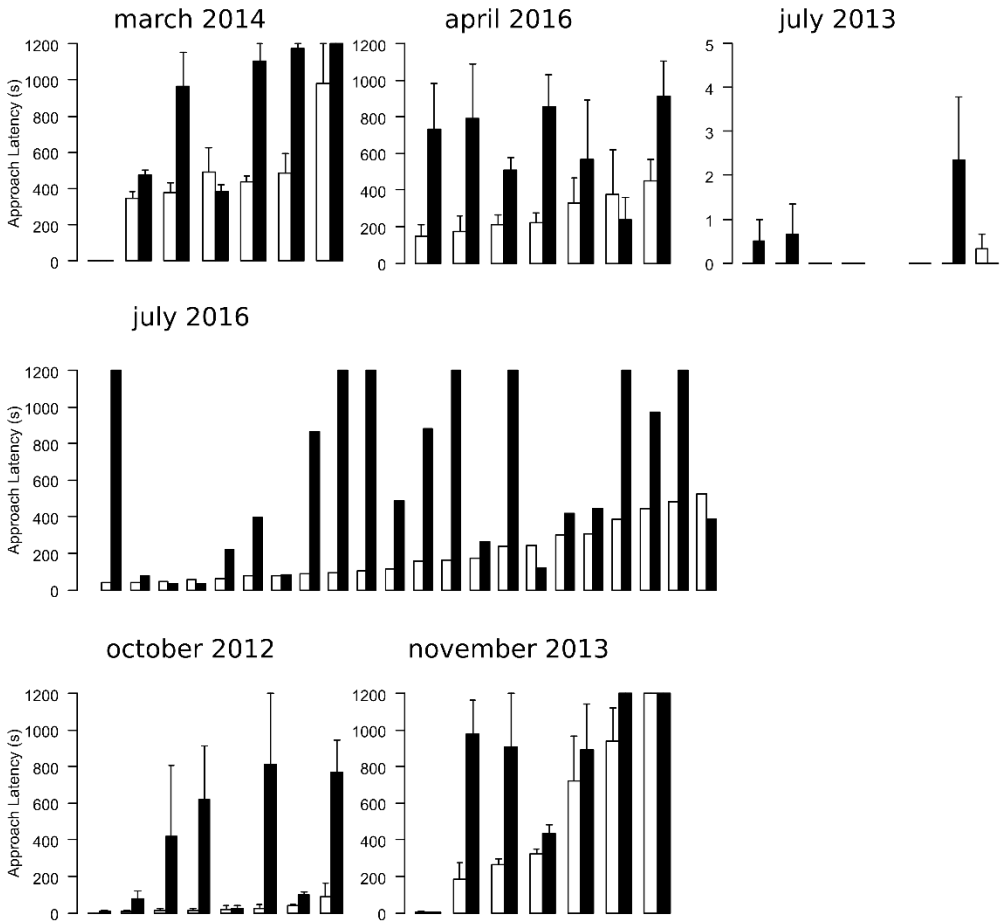

Figure 6.2. Individual variability in approach latency among house sparrows. Individuals are arranged by increasing time to approach the no object control. Approach latency of each bird was averaged for up to 3 trials for control (no object) and up to three trials for the novel object condition. Error bars indicate mean ± standard error. In July 2016, only one neophobia test was done per bird. White bars are controls; black bars are with novel object. Note that July 2013 has a very different y axis than the others.

CHAPTER 7: ARE NOVEL OBJECTS PERCEIVED AS STRESSFUL? THE EFFECT OF NOVELTY ON HEART RATE

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7.1 Abstract

Neophobia, or the fear of novel objects, is a behavior that is often found in wild animals. Neophobia appears to be related to the physiological stress response because individuals with higher glucocorticoid responses to stress often are more neophobic. The relationship between the heart rate response and novelty, however, has not been tested in a wild species. We implanted heart rate transmitters in captive European starlings (*Sturnus vulgaris*) to measure increases in heart rate as an index of the adrenomedullary stress response. Specifically, we measured heart rate in animals encountering novel objects on or near their food dishes using a system to display the novel objects while the experimenters remained outside the room, thereby minimizing the confounding effects of experimenter presence on heart rate. We analyzed three conditions: the period of adjustment to the experimental setup before any exposure to novelty, novel object trials, and no object controls (presented in a random order after 0-5 novel objects). Birds approached their food dishes faster during the adjustment period than during novel object trials. Although

they demonstrated a behavioral aversion to novelty, the effect on heart rate was unexpected. Heart rate increased sharply when the food dishes were displayed. The duration of the startle response was longer during no object controls than during novel object exposure, the opposite of the anticipated result. There were no correlations between behavior and metrics of the heart rate response. Novel object exposure does not cause an increase in heart rate.

7.2 Introduction

Neophobia, the "fear of the new", is an ecologically relevant behavior characterized by an aversion to novelty, particularly novel objects. To an animal in the wild, a novel object could represent either a threat or a potential untapped resource. A neophobic animal may avoid the risks associated with novelty such as predation (Brown *et al.*, 2013), but may not be able to gain access to new resources (reviewed by Greenberg and Mettke-Hofmann, 2001). Neophobia is often assessed by placing novel objects on or near a food source (Greenberg and Mettke-Hofmann, 2001). The animals must weigh the potential threat of the object against their desire to approach the food. Neophobia has been measured this way in a number of wild bird species in captivity (Apfelbeck and Raess, 2008; Boogert *et al.*, 2006; Greenberg, 1992; Martin and Fitzgerald, 2005) and in the field (Herborn *et al.*, 2010; Schoech *et al.*, 2009). In European starlings (*Sturnus vulgaris*) (Feenders *et al.*, 2011) and blue tits (*Cyanistes caeruleus*) (Herborn *et al.*, 2010) neophobic responses are equivalent in the wild and in captivity. Neophobic birds take much longer to approach a novel food dish or novel food type than they do their regular dish or food, but will rapidly habituate and no longer avoid novel objects as they

encounter them multiple times (Rankin *et al.*, 2009). Although fear of the new object may drive the behavioral responses, it is unclear whether the birds experience stress as they determine whether to approach an object they have never before encountered. The relationship between fear of novelty and stress is not fully understood.

The physiological stress response is a conserved reaction to a broad range of noxious situations that may be encountered by an animal, such as predator attacks, storms, disease, famine, etc. It consists of three arms. The adrenomedullary response begins in less than a second and consists of the increase in heart rate and mobilization of energy caused by the rapid release of catecholamine hormones (epinephrine and norepinephrine) from the adrenal medulla. The glucocorticoid response begins within minutes and can last for hours. It consists of the release of cortisol (in most mammals) or corticosterone (in birds) into the blood. Glucocorticoids have wide-ranging effects on many bodily functions, from energetics to immune function to reproduction to behavior (reviewed in Sapolsky *et al.*, 2000). The third branch of the stress response is the animal's adjustment of its behavior to appropriately confront the stressor. The three branches of the stress response are linked, but still maintain independent regulation from one another. For example, great tits (*Parus major*) show behavioral aversion to many stimuli, but they only increase glucocorticoid levels in response to some stimuli, i.e. a predator (Cockrem and Silverin, 2002). European starlings independently regulate their behavior, heart rate, and glucocorticoid levels in response to different types of stressful stimuli (de Bruijn and Romero, 2013; Nephew *et al.*, 2003). Neophobia

resembles the behavioral arm of the stress response – it is a behavioral response to a stimulus which an animal might perceive as threatening.

The relationship between the glucocorticoid arm of the stress response and neophobia has been assessed in several bird species, with mixed results. The glucocorticoid response to a standardized stressor correlated with the behavioral response to novel objects in free-living Florida scrub jays (*Aphelocoma coerulescens*) (Schoech *et al.*, 2009), free-living house sparrows (*Passer domesticus*) (Lendvai *et al.*, 2011), and great tits artificially selected for low neophobia (Baugh *et al.*, 2012). In these studies, birds with strong glucocorticoid responses also had high fear and/or low exploration towards novel objects. However, in free-living collared flycatchers (*Ficedula albicollis*) (Garamszegi *et al.*, 2012) and zebra finches (*Taeniopygia guttata*) artificially selected for high and low corticosterone responses (Martins *et al.*, 2007), there was no relationship between glucocorticoids and behavior towards novel objects. Novel objects cause either no or a very small glucocorticoid response in both European starlings (Apfelbeck and Raess, 2008) and Japanese quail (*Coturnix japonica*) (Richard *et al.*, 2008) even when the birds demonstrate a strong behavioral response. In our previous experiments, an injection of corticosterone had no effect on neophobia in European starlings (unpublished data, D. Merullo and R. DeBruijn). In sum, it appears that there is a connection between neophobia and the glucocorticoid response, but it is still unclear whether neophobia activates the glucocorticoid arm of the stress response in birds. Furthermore, the time frame of a neophobic response and glucocorticoid release are not well matched.

In contrast, neophobic behavior and the heart rate response share a similar time frame that is much more rapid than glucocorticoid release, so perhaps neophobia activates the adrenomedullary arm of the stress response. The heart rate response to stress can be measured in European starlings using implantable heart rate transmitters (Cyr *et al.*, 2009; Nephew *et al.*, 2003). Captive starlings show a robust startle response (i.e., a rapid increase in heart rate) within a fraction of a second of being exposed to various types of stressors (Cyr *et al.*, 2009; Nephew *et al.*, 2003). In this study, we tested the hypothesis that novel objects will cause an increased heart rate in starlings. We measured heart rate in captive wild-caught European starlings during novel object exposure or exposure to the birds' normal food dish. Because the presence of a human causes a strong heart rate response in starlings (Nephew *et al.*, 2003), we created a system to expose hungry animals to either their familiar food dish or a dish modified with a novel object while the experimenter remained outside the room. We hid the food dish in an opaque black box that we lifted by pulling a string that ran outside the room where the birds were housed. When the black box was suddenly lifted, we anticipated that the animals would have a startle response (i.e., a sharp increase in heart rate). We hypothesized that heart rate would take longer to return to baseline when the animals were exposed to a novel object instead of their familiar dish. That is, the combined effect of startle and novelty would be greater than the startle effect alone. We further hypothesized that the animals would approach the food dish at approximately the time that their heart rate returned to baseline.

7.3 Methods

7.3.1 Animals

Twelve European starlings were caught in March 2014 at a suburban dairy farm in eastern Massachusetts. They were housed in an outdoor aviary until late May 2014. They were then moved to an indoor animal facility and housed in individual cages on a 16L:8D light cycle. They were given at least four weeks to acclimate to the indoor facility. Heart rate could be measured on only four birds at a time. Two groups of four were tested in June-July; one group of four was tested in September. The birds from September had been used as controls in a previous experiment. Feather and blood samples had been taken from these birds one month or more before the beginning of this trial, but otherwise they were only exposed to standard caretaking. Just before experiments began, they were surgically implanted with heart rate transmitters and allowed to recover for several days (following Nephew *et al.*, 2003). Heart rate transmitters were purchased from Data Sciences International (St. Paul, MN). We used two different models, TA 10EA-F20 and TA 11ETA-F10. The transmitters send radio signals to a receiver attached to 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. The heart rate transmitters have a magnetic switch which could be turned on at the beginning of every experimental day. The birds' weights were monitored 1-2x weekly throughout the experiment. No significant weight changes were observed. We used a mix of males and females. There have been no previous differences found between the stress responses of captive males and females in this species (Nephew and Romero, 2003; Nephew *et al.*, 2003;

Nephew *et al.*, 2005). All experiments complied with Association for Assessment of Laboratory Animal Care guidelines and were approved by the Tufts Institutional Animal Care and Use Committee.

7.3.2 Remote presentation of objects

Because we were looking for the heart rate response to novelty, we wanted to reduce the heart rate response to extraneous stimuli as much as possible. Therefore, we constructed a device to allow the animals to be exposed to novel objects without the presence of the researchers (Figure 7.1). A black box constructed of foam core board was placed over the food dish. It was hinged onto the cage to prevent birds from entering from the top and was weighted with washers to prevent the birds from lifting it up. A string allowed researchers to pull the box up and display the food dish from outside the room. The behaviors of the animals were monitored using video cameras.

7.3.3 Neophobia trials

On the night before each experimental trial, the birds' food dishes were removed overnight to stimulate appetite and ensure the animals would be motivated to approach their food dishes in the morning. Opaque blinders were placed between the cages at the same time to prevent the birds from seeing one another's behavior and the objects the other birds were being exposed to. Starlings show more neophobia in isolation than when they are in contact with other starlings (Apfelbeck and Raess, 2008). However, an individual bird's response to novelty can be affected by the behavior it has observed in others (Fryday and Greig-Smith, 1994). In the morning, birds were caught and their heart rate transmitters were switched on. At

the same time, the opaque boxes were placed in the cages over the animals' food dishes (birds were restrained in a cloth bag for ~5 minutes while boxes were placed). The animals were then given 10 minutes for their heart rate to recover from handling stress. Baseline heart rate was then assessed for 20 minutes (beginning t=-20). At t=0, the box was lifted, exposing birds to their food dish. Behavior and heart rate were monitored for 20 minutes. Heart rate transmitters were then switched off, boxes and novel objects removed from the cage, and the opaque dividers removed from between the cages. The birds were acclimated to this procedure by presenting the normal food dish for at least three days before the start of the experiment. (The first four birds tested in June were given 10 days to adjust; due to time restrictions, the remainder of the birds were given 4-5 days.)

On experiment days 1 through 7, birds were exposed to novel objects in a random order. The objects used were unlikely to resemble anything the birds had previously encountered. Objects used were a food dish painted red, a yellow bottle opener keychain clipped to the cage behind the food dish, a colored plastic Easter egg placed in the food dish, a springy red ring wrapped around the food dish, a red ribbon stretched across the top of the food dish, and a white cover placed over the food dish. Many of the objects were red because that has been shown to be a color that more powerfully elicits neophobia (Roper, 1990) and each object had been previously shown to stimulate neophobia in European starlings (unpublished data, D. Merullo and R. DeBruijn). Birds were also shown a no object control (i.e., normal food dish) on one randomly chosen experimental day.

Videos were analyzed for the latency to approach and feed from the dish from the time the box was lifted. Approach was defined as perching on the dish for at least 1 second; feeding was defined by the beak dipping into the dish for at least 2 seconds. Different objects may stimulate neophobia to a greater or lesser degree, but we were not interested in the differences between objects, just in an individual's mean response to novelty. We therefore averaged the approach and feed latency times for each bird across all novel objects.

Heart rate was measured in the animals for 20 minutes before and 20 minutes after object exposure. It was analyzed using the Ponemah Physiology Platform (Data Sciences International, Valley View, OH). Heart rate (beats per minute, bpm) was calculated in thirty second intervals using a forward frame (i.e. averaged over the 30 seconds following each time stamp). Some parts of the traces contained breaks in the data or noise which disrupted the signal. The traces were each individually examined, and problematic portions were eliminated manually. Maximum heart rate was determined as the highest bpm after raising the box, and the duration of the startle response was calculated as the time for the heart rate to return to within 1 standard deviation of baseline (Nephew *et al.*, 2003).

7.3.4 Data analysis

All statistical analyses were conducted in R (Version 3.1.3; R Core Team). The design for some of our analyses was unbalanced (for example, heart rate traces were sometimes unusable), so linear mixed effects models were constructed using the "lmer" function in the lme4 package of R (Bates *et al.*, 2015) with individual bird as a random variable. We then used the "Anova" function in the car package

(Fox and Weisberg, 2011) to calculate a Type II Wald F test with Kenward-Roger adjusted degrees of freedom. We followed this with a Tukey's multiple comparison test if warranted. This method was used for all analyses. Approach latency and feed latency were analyzed by averaging the values over all novel object trials and comparing the resultant means to the no object trials, An unanticipated behavioral difference was detected between the adjustment period and the experimental trials. We therefore analyzed behavioral and heart rate metrics (approach latency, feed latency, baseline heart rate, peak heart rate, and duration of startle response) by comparing the adjustment period, the no object controls, and the trials with novel objects. All data are reported as mean ± standard error.

7.4 Results

7.4.1 Behavior

Of the twelve birds in the study, three birds did not approach the no object control during the experimental period. (See Figure A1.1A for individual approach latencies.) These three birds were excluded from behavioral analyses. We compared mean latency to approach the novel dishes with latency to approach the familiar dish and found a significant effect of novelty ($F_{1,8}$=5.48, p=0.047, Figure 7.2A). Similarly, there was a significant effect of novelty on mean feed latency ($F_{1,8}$=6.50, p=0.03, Figure 7.2B). Birds took 362 ± 123 seconds (mean±se) to approach the familiar dish, and 589 ±89 seconds to approach a novel dish. They fed from the familiar dish after 495±146 seconds and from a novel dish after 798±106 seconds. Data from all birds (including those that did not approach the no object dish) are presented in Figure 7.8.

The birds showed a moderate response to novelty. However, they behaved differently during the experimental period compared to when they were adjusting to the experimental setup. Behavior and heart rate was only recorded for the last 1-3 days of the adjustment period (adjustment period lasted 4-10 days). Animals took significantly less time to approach during the adjustment period than during the novel object exposure (Overall model: $F_{2,60.0}$=7.07, p=0.002; Tukey's: adjust vs. novel object: z=3.65, p<0.001, Figure 7.3A). They fed more quickly during the adjustment period than during novel object exposure (Overall model: $F_{2,60.1}$=6.04, p=0.004; Tukey's: adjust vs. novel object: z=3.24, p=0.004, Figure 7.3B). The number of times the birds were exposed to the box did not affect the time to approach or feed (Approach: $F_{1,27.7}$=0.72, p=0.40, Figure 7.3C; Feed: $F_{1,24.5}$=1.53, p=0.23, Figure 7.3D). These patterns held true when the three birds that did not approach during experimental no object trials were included in the analysis (see Figure 7.9).

7.4.2 Heart rate

Of the twelve birds, only nine had usable heart rate data. There was no difference in baseline heart rate (averaged over the 10 minutes before the box was lifted) throughout the experiment (adjustment period: 338±13 bpm; no object control: 343±18 bpm; novel dish exposure: 358±10 bpm; $F_{2,53.7}$=1.12, p=0.33). When the box was lifted to expose animals to their food dish, the birds had a strong startle response (Figure 7.4). Heart rate sometimes may have increased just before box lift when birds could hear the boxes move in the neighboring cages. There was no significant difference in maximum startle-induced heart rate between the

adjustment period, the no object controls, and the novel object exposures ($F_{2,53.9}$=1.74, p=0.19, Figure 7.5A). There was a significant difference in the duration of the startle response between adjustment, no object, and novel object ($F_{2,55.4}$=3.65, p=0.03, Figure 7.5B). During the no object trials, duration was marginally longer than during the adjustment period (z=2.21, p=0.07) and significantly longer than during the novel object exposure (z=-2.66, p=0.02). There was no difference between the adjustment period and novel object exposure (z=-0.095, p=1). (See Figure 7.7B for individual startle durations.)

Approach time and feed time were unrelated to the duration of the startle response (Approach: $F_{1,57.6}$=1.37, p=0.25, Figure 7.6A; Feed: $F_{1,58.6}$=1.06, p=0.31, Figure 7.6B). Approach and feed times were also unrelated to maximum heart rate (Approach: $F_{1,61.8}$=0.35, p=0.56, Figure 7.6C; Feed: $F_{1,62.0}$=0.02, p=0.88, Figure 7.6D). On average, the startle response was completed more than a minute before the birds approached the food dish (Figure 7.4).

7.5 Discussion

Neophobia is an ecologically relevant behavior in wild birds. Different ecological and behavioral contexts require a different balance of caution and boldness. Individuals within a species or across species use different strategies and therefore have different levels of neophobia. Less fear may be more beneficial for species which employ a generalist strategy (i.e., those which eat a broad variety of foods) or are currently invading new territory (which contains unknown food sources), as they will come into contact with novelty more frequently and have more to gain by seeking out new resources (Echeverria *et al.*, 2006; Greenberg and

Mettke-Hofmann, 2001). For example, house sparrows that are currently invading new territory demonstrate less neophobia than house sparrows which have occupied the same region for several generations (Martin and Fitzgerald, 2005). Similarly, the habitat-generalist song sparrows are less neophobic than their specialist relatives, the swamp sparrow (Greenberg, 1990; Greenberg, 1992). Individuals tend to adapt a stable behavioral strategy for coping with the environment. Neophobia is a key part of these strategies. Individual birds tend to show repeatable levels of neophobia when shown different novel objects (Greenberg, 1990; Schoech *et al.*, 2009). Some measure of neophobia is commonly used when assessing different personalities which are stable in different contexts (Boogert *et al.*, 2006; Drent *et al.*, 2003). Nevertheless, the physiology behind the behavior is not fully understood. Does avoidance of novel objects indicate that an individual finds their presence stressful?

A few previous experiments have examined the relationship between the heart rate response and neophobia. Horses exposed to novel audiovisual stimuli (a film being played while the animals exercised on a familiar treadmill) showed an increase in heart rate as well as increased ACTH (a hormone involved in the glucocorticoid pathway) (Hada *et al.*, 2003). Rats showed an increase in heart rate when exposed to the novel environment of an open field (van den Buuse *et al.*, 2001), although much of the difference in heart rate can be explained by a change in wakefulness – the nocturnal animals were exploring the field in the middle of the day during a time in which they are normally asleep (Beerling *et al.*, 2011). Two lines of great tits (*Parus major*) have been artificially selected for high or low levels

of neophobia (Drent *et al.*, 2003). The high neophobia line had a higher breath rate in the hand than the low neophobia line, which provides a rough estimation of heart rate during a stress response (Carere *et al.*, 2001). Our current study is the first test of object neophobia in a wild bird species that measured heart rate changes.

Measuring heart rate responses to new objects in wild birds presented some unique challenges. In previous experiments, novel objects were presented directly to the animal by the experimenter (Boogert *et al.*, 2006; Greenberg, 1992; Herborn *et al.*, 2010). However, that was unfeasible in our study – any subtle effect the novel object had on heart rate could be masked by the strong effect of experimenter presence (Cyr *et al.*, 2009). We therefore used a black opaque box to hide the food dish and novel objects from the birds while we recorded baseline heart rate. The box was lifted up with a string from outside the room at t=0, leaving the box dangling over the food dish. The movement of the box itself caused a heart rate response. However, its use eliminated some sources of variability that would be caused by the experimenter directly presenting the food dishes (eg. identity of the experimenter, length of time in the room, amount of time to position the objects.) During the adjustment period, every bird was observed to approach and feed at least once.

The animals in our study showed only moderate neophobia. Three birds never approached their food dish in the twenty minute observation period during experimental trials. These individuals were likely temperamentally different from the other nine birds. Individuals can vary in the intensity of their neophobic responses, at least in house sparrows (Ensminger and Westneat, 2012). When these

three birds were removed from behavioral analyses, we could detect a small behavioral difference between the no object controls and the experimental controls. However, a much more robust behavioral difference was found between the initial period of adjustment to the box and the experimental trials. The birds approached and fed with the box much more readily during the last few days of the adjustment period than they did during novel object exposure, a result that was robust whether or not the three temperamentally different birds were included (Figs. 3 and S3). During the adjustment period, many animals appeared unafraid of the box and tried to get inside (personal observation). Because of their persistent efforts, the box had to be weighted heavily with metal washers to prevent them from moving it. However, once they had been exposed to novelty, they perhaps became more wary of the setup as a whole. Some birds discriminated between no object and novel object even after being exposed to novelty. Other birds took longer to approach even the no object controls once they had been exposed to a novel dish – blunting the effect of novelty on behavior. The birds may have learned to be cautious of the box once a novel dish had been revealed – it was no longer certain what would be revealed when it was lifted up. The number of times the birds were exposed to the box did not affect their behavior, either during the adjustment period or during experimental trials. However, the first exposure to the box was never recorded.

In addition to the box in their cages, the birds were also exposed to the stress of handling before the experiments. Although neophobia does not appear to be stressful in itself, the stress response can affect neophobia. In previous trials using European starlings, exposure to restraint stress before novel object exposure

increased the latency to approach the object (unpublished data, D. Merullo and R. DeBruijn). Because of the procedure for measuring heart rate, the animals in this study necessarily experienced stress before novel object exposure, which may have resulted in more intense neophobia. The stress of handling may have increased the learned fear of the box once novelty had been experienced. Elevated corticosterone has been implicated in learning and memory. For example, mountain chickadees (*Poecile gambeli*) with chronic but moderate elevation of corticosterone had improved spatial learning (Pravosudov, 2003). Handling may therefore have been partly responsible for the increase in latency to approach the no object controls during the experimental period. Increased corticosterone levels resulting from handling may have helped the animals learn to fear the box once it was shown to be unpredictable by the introduction of novelty.

Of the twelve birds, we were able to get usable heart rate traces from nine. We included all birds in the heart rate analysis whether they approached the no object controls or not because the more robust behavioral difference was between the adjustment period and novel object exposure, a difference that was observed in all birds. The birds all had a startle response when the box was lifted. We measured the combined effect of startle and novelty – that is, how novel objects affected the duration and magnitude of the startle response. Despite the behavioral differences between the trials, the strength of the heart rate response was not different between the adjustment period, the novel dish exposure, and the experimental no object exposure. The startle response lasted an average of 2 minutes during the adjustment period or novel object exposure. However, during the no object control trials, heart

rate took longer to return to baseline, about 3.3 minutes. The birds that did not approach the dish during the no object trials, or that had a longer latency to approach than during novel object trials, tended to have particularly high heart rates during no object trials (see birds 446.M, 481.M and 493.M in Figure 7.7). For some individuals, encountering a normal dish when they expected to see a novel object may have led to a stronger fear response. We did not have a large enough sample size to address the question of why some animals were more fearful of the no object dish than others. On average, by the time the animals approached the food dish, their startle response had been completed for several minutes. There was no correlation between the duration of the startle response and the time to approach or feed from the food dish. There was also no correlation between the maximum heart rate and either metric of behavior.

In conclusion, we did not find a strong neophobic response in these birds. However, exposure to novelty affected the birds' behavior – once exposed to a novel object, they approached their dishes more slowly. We anticipated that the birds would show a startle response (increased heart rate) when they were exposed to their food dishes, but hypothesized that the duration of the startle response would be longer with a novel object then without one, corresponding to a longer latency to approach novel food dishes. If the heart rate response is related to the behavior of neophobia, we would expect the startle response and the fear of the food dish to resolve at approximately the same time. We rejected this hypothesis. The heart rate response to startle was completed well before the animals approached and fed from their dishes and there were no correlations between startle response metrics and

behavior. It thus appears that captive European starlings do not have a robust adrenomedullary stress response when exhibiting neophobic behavior.

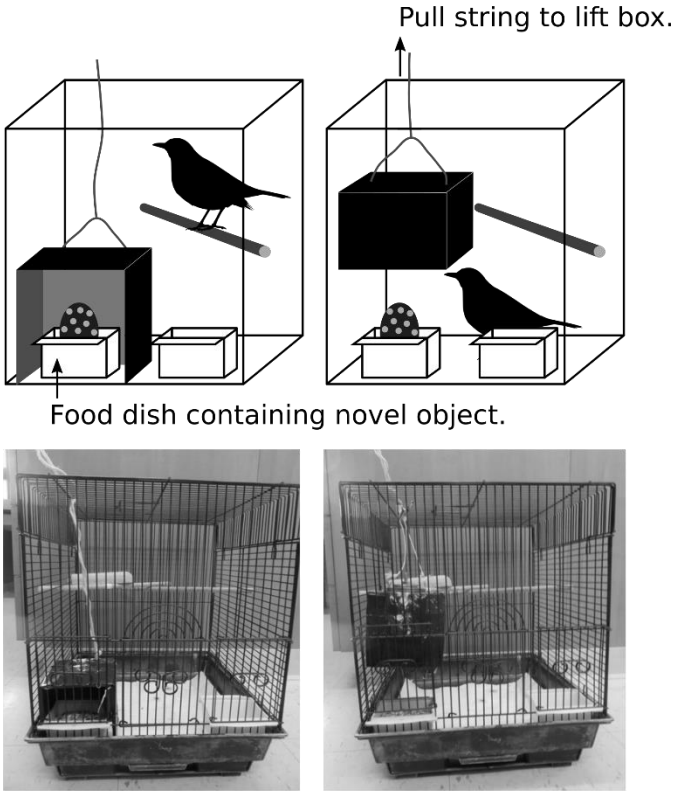

Figure 7.1 Device to remotely show novel objects. Researchers can lift up the opaque black box by pulling a string from outside the room.

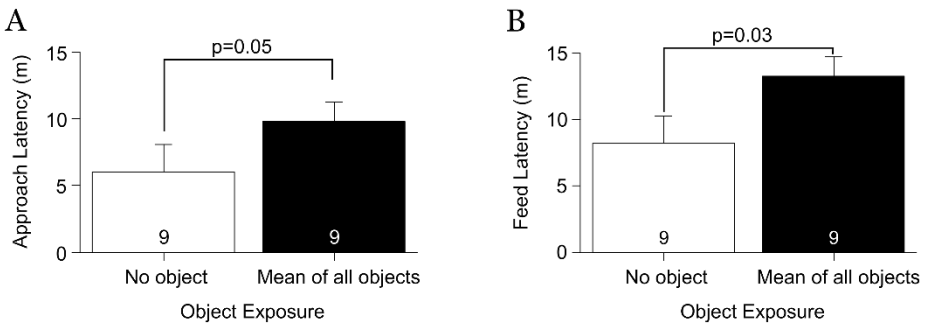

Figure 7.2 Neophobic responses in European starlings to novel objects on food dishes. A) Time to approach the feed dish for the no object control vs the mean time to approach novel object exposure for each bird. B) Time to feed from the dish for the no object control vs. the mean time during novel object exposure for each bird. Error bars indicate mean+SE. Data are from the birds that approached the unaltered dish. Data for all birds is shown in Figure 7.8.

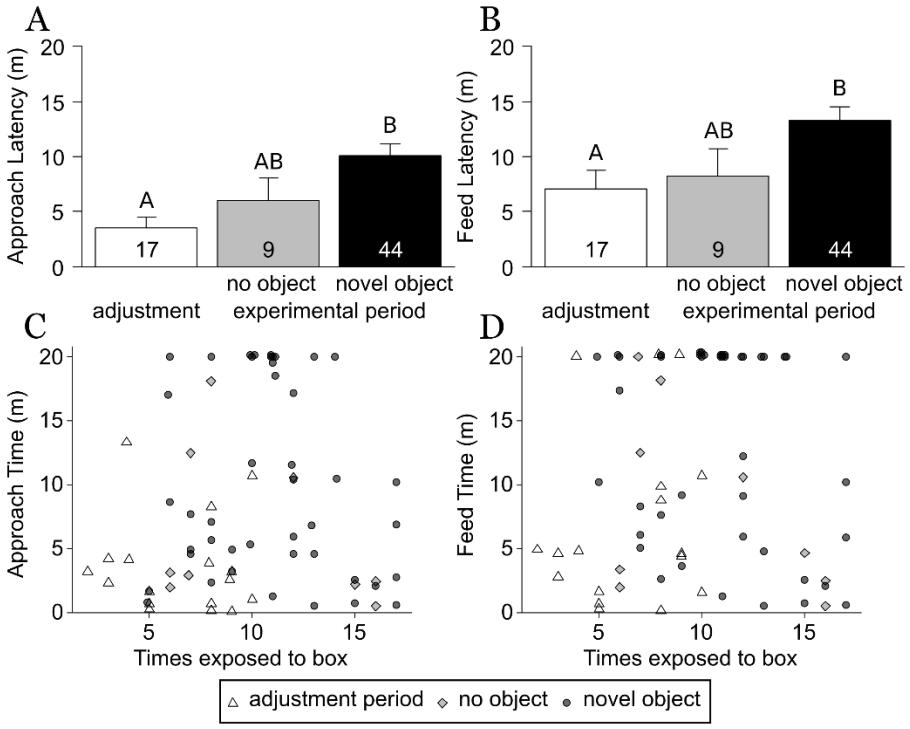

Figure 7.3 Approach and feed time during adjustment period, no object control, and novel objects trials. A) Birds took longer to approach the feed dish during novel object exposure than during the last three days of the adjustment period. B) Birds took longer to feed from the dish during novel object exposure than during the adjustment period. Error bars indicate mean + SE. Different letters indicate significant differences. There is no relationship between approach time (C) or feed time (D) and the number of times exposed during the adjustment period or the experimental period. The first time the birds were exposed to the box was never recorded. Data are from the birds that approached the unaltered dish. Data for all birds is shown in Figure 7.9.

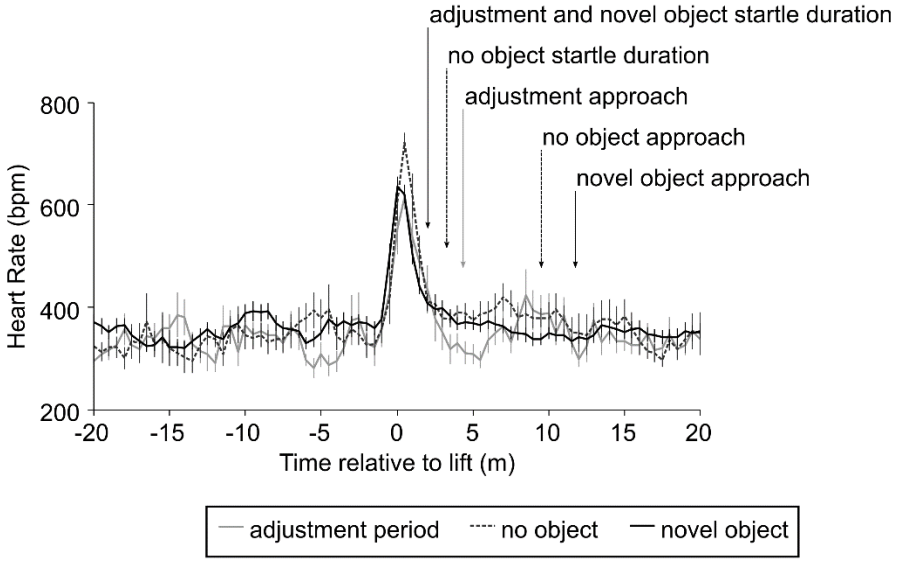

Figure 7.4 Heart rate during the adjustment period, the no object controls, or exposure to novel objects. Birds were exposed to the food dish at t=0. Arrows indicate the mean time for heart rate to return to baseline (startle duration) and mean time to approach the food dish. Startle duration during the adjustment period and novel object exposure were equivalent. Birds approached the novel object well after the end of the startle response. Heart rate was averaged using a "forward" frame – i.e. average of 30 seconds following each time point.

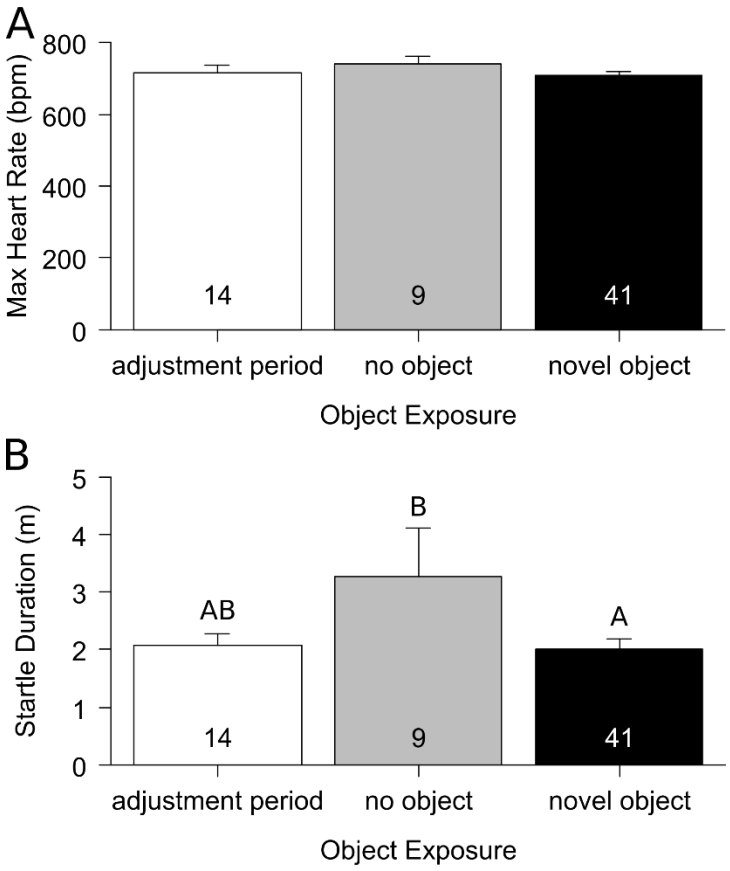

Figure 7.5 Startle response during the adjustment period, exposure to the normal food dish, or food dish plus novel object. A) Highest heart rate after dish exposure. B) Time for heart rate to return to within 1 SD of baseline. Different letters indicate significant differences. Error bars indicate mean ± SE.

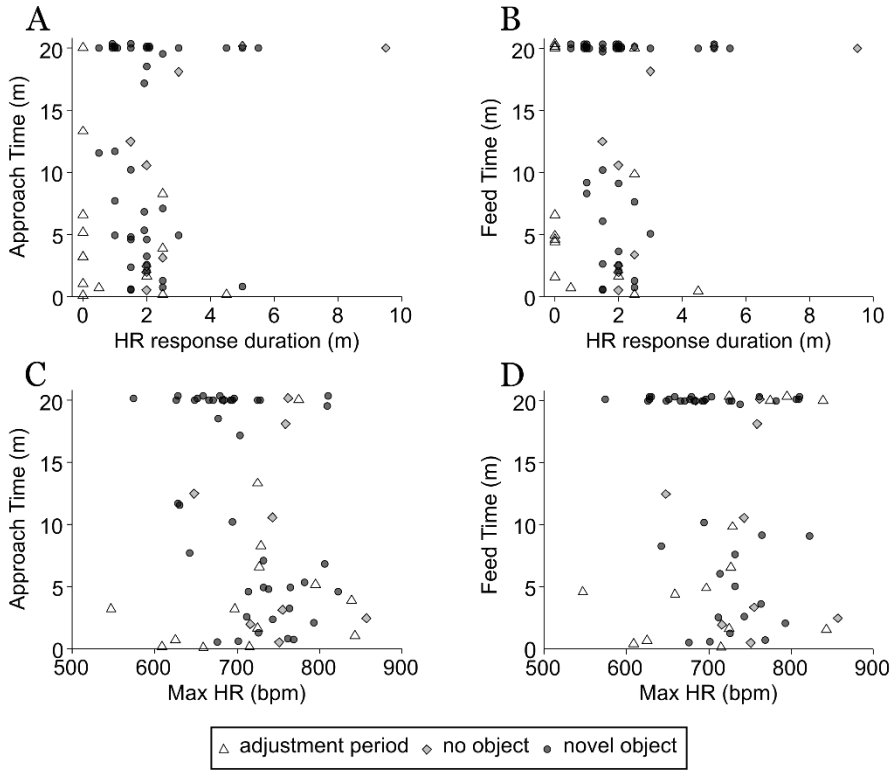

Figure 7.6 There is no relationship between heart rate response and neophobic behavior. Heart rate response duration (time to return to baseline after startle) vs. approach time (A) and feed time (B). Maximum heart rate after startle vs. approach time (C) and feed time (D).

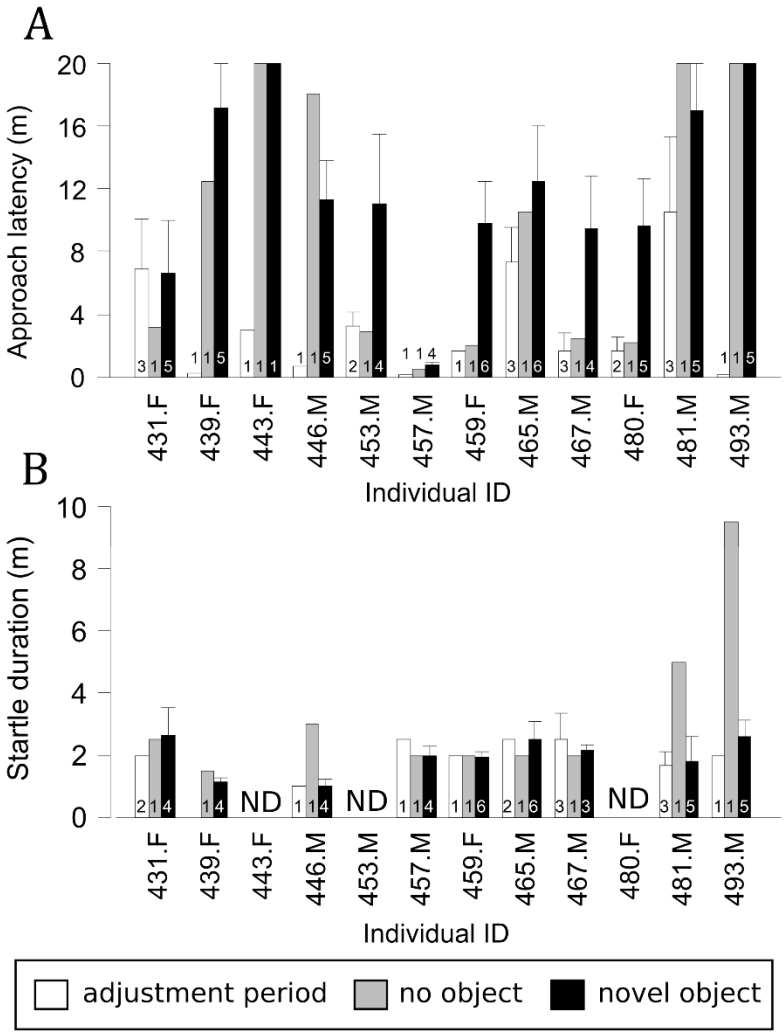

Figure 7.7. Individual variation in approach latency (A) and heart rate response duration (B). ND=no data. Error bars indicate mean + SE. Numbers in the bars indicate the number of trials recorded. Bird IDs ending in ".F" are female, those ending in ".M" are male.

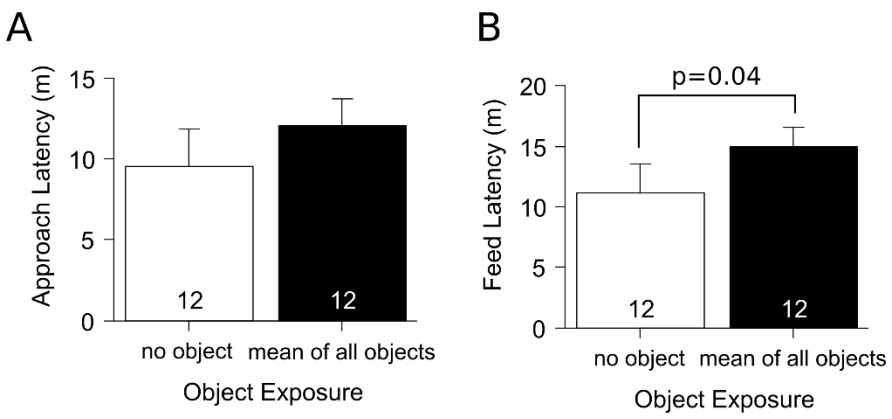

Figure 7.8. Neophobic responses in European starlings, all twelve animals included. A) Time to approach the feed dish for the no object control vs the mean time to approach novel object exposure for each bird ($F_{1,11}$=3.59, p=0.08). B) Time to feed from the dish for the no object control vs. the mean time during novel object exposure for each bird ($F_{1,11}$=5.55, p=0.038). Error bars indicate mean+SE.

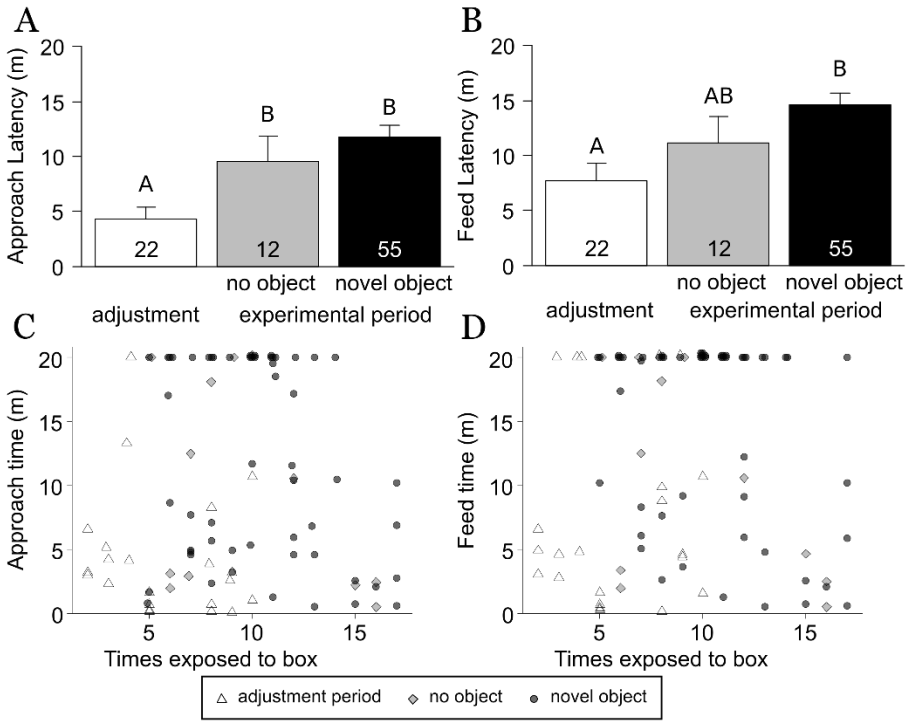

Figure 7.9. Approach and feed time during adjustment period, no object control, and novel objects trials, all twelve animals included. A) Birds took longer to approach the feed dish during the experimental period than during the last three days of the adjustment period (Overall model: $F_{2,76.2}$=11.42, p<0.0001; Tukey's: adjust vs. novel object: z=4.79, p<0.001; adjust vs no object: z=2.35, p=0.05). B) Birds took longer to feed from the dish during novel object exposure than during the adjustment period (Overall model: $F_{2,76.4}$=9.68, p=0.0002; Tukey's: adjust vs. novel object: z=4.36, p<0.0001). Error bars indicate mean + SE. Different letters indicate significant differences. There is no relationship between approach time C) or feed time D) and the number of times exposed during the adjustment period or the experimental period (Approach: $F_{1,52.5}$=1.42, p=0.24; Feed: $F_{1,43.4}$=2.51, p=0.12). The first time the birds were exposed to the box was never recorded.

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APPENDIX I: EXOGENOUS AND ENDOGENOUS CORTICOSTERONE IN FEATHERS

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Abstract

In birds, the steroid hormone corticosterone (CORT) increases in response to real or perceived threats to homeostasis. A long-term record of CORT exposure is recorded in feathers when the hormone is incorporated into the keratinized tissue, and then perserved when the mature feather is cut off from the blood supply. The opportunity to retrospectively assess the exposure of an individual to stressors by measuring the amount of CORT in a feather has generated excitement amongst avian ecologists. However, this technique is relatively new and requires additional validations. In this study, we performed experiments in wild caught European starlings (*Sturnus vulgaris*) to test 1) whether CORT deposition in the feather depends on time of day, 2) CORT that is locally applied to the skin near the growing feathers is reflected in feather CORT, rather than CORT delivered to the feather through the circulation, 3) whether an experimentally applied but ecologically relevant stressor (food restriction) causes a change in feather CORT. We found that exogenous CORT was incorporated into feathers during the day and the night. Feather CORT most likely represents circulating plasma CORT – therefore CORT synthesized in skin is likely to have a negligible effect on feather CORT. However, there was no difference in feather CORT between birds that were food restricted and those that were not, indicating that feather CORT might not always detect ecologically relevant stressors.

1 Introduction

The glucocorticoid response to stress is a conserved physiological reaction in vertebrates. Glucocorticoid hormones (corticosterone, CORT, in birds) are secreted from the adrenal cortex in a baseline diel rhythm which then increases in response to threats to homeostasis. CORT can become incorporated into keratinized tissue, such as hair, nails, or feathers. In 2008, Bortolotti and colleagues developed an assay to extract CORT from feathers. CORT in feathers is thought to provide a record of the sum total of plasma CORT exposure during the period of feather growth. In theory, an animal that experienced more stress during the time the feather was growing will have higher CORT in its feathers than a less stressed individual.

Bortolotti *et al.*'s assay has been gaining increasing interest in the past few years. However, it is a relatively new technique and requires additional validation work (Romero and Fairhurst in press). Previous work in European starlings

(*Sturnus vulgaris*) showed that molting birds use more energy at night, which may indicate that feather growth occurs preferentially at night (Cyr *et al.* 2008). If that is the case, then hormones may be incorporated more at night than during the day. Feather CORT may be reflective of nighttime levels of circulating CORT, which is the diel peak in this (Romero and Remage-Healey 2000) and other avian species (e.g. Rich and Romero 2001; Breuner *et al.* 1999). In our first experiment, we administered exogenous corticosterone in the morning or the evening and compared levels of CORT in the feathers. We applied CORT in dimethyl sulfoxide (DMSO) to the skin of European starlings that were regrowing feathers that had been experimentally plucked. DMSO carries the CORT through the skin and elevates circulating CORT for a brief time compared to the days or weeks long elevation caused by CORT implants (Busch *et al.* 2008). We expected feather CORT levels to be greater in birds that received exogenous CORT at night compared to those that were treated during the day.

Another potentially confounding factor in analyzing feather CORT analysis is the possibility that avian skin may produce its own CORT. Mammalian skin can locally produce CORT that may not go into general circulation or affect other tissues (Slominski *et al.* 2007). Although it is not known whether birds also have this capacity, it is possible that locally produced CORT is incorporated into feathers. If so, then feather CORT may represent locally produced CORT and not circulating plasma CORT. We simulated locally produced CORT by applying our treatment topically to the skin near the feather tracks of the growing feathers on one wing only. If feather CORT is representative of circulating and not locally produced CORT, we would expect no difference between the feathers from the wing where CORT was applied and the other wing.

Several studies have found that if exogenous CORT is given during feather growth, it will result in higher feather CORT (Fairhurst *et al.* 2013; Jenni-Eiermann *et al.* 2015; Lattin *et al.* 2011). Many more studies have made use of feather CORT in an ecological context, relating feather CORT to, for example, food quality, immunity, sexual coloration, or carry over effects between life history stages (Harms *et al.* 2015; Lendvai *et al.* 2013; Sild *et al.* 2014; Will *et al.* 2015). However, there have been relatively few studies where experimentally induced stress has been related to feather CORT levels. In our second experiment, we exposed captive European starlings to an ecologically relevant stressor during the period of feather regrowth and measured CORT in feathers of stressed and unstressed birds. We induced chronic stress using a random food removal protocol. Food dishes were removed for four hours each day at a random time. The animals were not calorically challenged; they were provided *ad libitum* food for 10 hours during the light period. However, the unpredictable, uncontrollable removal of food dishes has been shown to induce chronic stress in European starlings (Bauer *et al.* 2011; Strochlic and Romero 2008) and other avian species (Freeman *et al.* 1981; Kitaysky *et al.* 2005; Lynn *et al.* 2003). In some studies, baseline and/or stress induced plasma CORT decreased with food removal (Kitaysky *et al.* 2005). In others, baseline or stress induced plasma CORT increased (Freeman *et al.* 1981; Lynn *et al.* 2003; Strochlic and Romero 2008) or did not change (Bauer *et al.* 2011). We expected to see a difference in feather CORT between treatment groups.

2 Methods

2.1 Experiment 1 – exogenous CORT

24 European starlings were collected at a suburban dairy farm in eastern Massachusetts in March 2014. Birds were housed in an outdoor aviary until May 2014. They were then moved to individual cages in an indoor facility with a 14L:10D light cycle (lights on 6AM, off 8PM). They were given four weeks to acclimate to the indoor conditions.

2.1.1 Exogenous CORT validation

We first tested the method of administering a short treatment of CORT using DMSO, a nonpolar carrier solvent. We dissolved crystalline CORT (Sigma, C2505) in DMSO and applied it to an area of skin plucked free of feathers. Twelve birds were divided into three treatment groups: no treatment, 20μL DMSO, and 20μL of 1mg/ml CORT in DMSO. We took a blood sample within 3 minutes of disturbance, then treated the birds and left them in their home cages. We took further blood samples at 30, 60, and 120 minutes. Plasma CORT concentrations were then measured (see below).

2.1.2 Experimental design

On day 1, all 24 birds were briefly anesthetized with isoflurane. Primary flight feathers 4-7 (P4-7) and secondary flight feathers 1-3 (S1-3) were plucked from each wing to simulate molt, following Strochlic and colleagues (2008). A small patch of body feathers was also plucked from the chest as part of a different experiment. By plucking many feathers at once, we ensured that feather growth would be an energetically costly process compared to re-growing just one feather. We were therefore more likely to observe the catabolic effects of corticosterone on feather replacement (Strochlic and Romero 2008). The S2 feather from each wing was stored for future analysis.

The birds were divided into three groups of 8: No CORT, AM CORT, and PM CORT. The No CORT group was left untreated. The AM CORT group was treated daily with CORT in DMSO between 8 AM and 10 AM. PM CORT birds were treated daily between 6 PM and 10 PM. In CORT treated birds, the feather coverts on the left wing were plucked to create a bare stretch of skin along the feather track of the growing flight feathers. 20μL of 1mg/ml CORT in DMSO was applied to this patch of skin. Experimental administration of CORT began on day 7 following feather removal. This lag represents the approximate emergence of pin feathers and allowed the birds to mobilize nutrients and prepare for feather regrowth.

CORT administration continued until all birds had regrown all their flight feathers, approximately the 6^{th} week after plucking. Feather growth (rate or length) was not measured for individual birds. The S2 feathers were regrown between week 4 and week 5, but the body feathers took longer to regrow, and in some cases, did not regrow at all. Birds were weighed three times a week to ensure that they maintained a stable weight. Birds never lost more than 15% of their starting mass.

On day 48, the regrown S2 feather was plucked from both right and left wings and analyzed for CORT (see below).

2.2 Experiment 2 – seminatural stressor

13 European starlings were collected in March 2015 from a suburban dairy farm in eastern Massachusetts. They were housed in individual cages at an indoor facility for four weeks to acclimate to captive conditions before the experiment began.

2.2.1 Plasma CORT

Before the beginning of the experiment (on day -3 for half the birds, on day -1 for the rest), plasma samples were taken to determine each individual's physiological stress response. A baseline sample was taken within three minutes of disturbance, when plasma CORT still reflects pre-disturbance levels and has not begun to rise (Romero and Reed 2005). Birds were then restrained in a cloth bag, which is a standard stressor to stimulate a CORT response (Nephew and Romero 2003). After 30 minutes, another blood sample was taken, after which a dexamethasone (DEX) suppression test was performed (Rich and Romero 2005). Birds were injected with 1mg/kg DEX and a final blood sample was taken 90 minutes later. Plasma CORT concentrations were analyzed as below. A second stress series was performed on day 39 or 41 at the end of the experiment.

2.2.2 Experimental design

On day 0, all birds were subjected to a simulated molt. Birds were anesthetized with isoflurane. P5-7 and S2-4 were plucked from each wing. The P7 feather from the birds' left wing was stored for analysis. Birds were randomly sorted into control (n=6) and food restricted (n=7) groups. We removed the food dishes from the food restricted birds for four hours daily. Four hours of food removal was chosen to maintain the birds at greater than 90% of their pretreatment weight (Strochlic and Romero 2008). To determine when during the day food was removed, we divided the hours from 8 AM – 10 PM into four-hour blocks. Each time block was assigned a number between 1 and 6, and a random number generator was used to determine which period food would be removed from the birds for each day. When not removed, food was available *ad libitum*.

Birds were weighed on days 0, 14, 21, 29, 32, and 35. Birds never lost more than 15% of their starting mass. By day 38, all P7 feathers had regrown. P7 from the left wing was plucked and saved for analysis and the food restriction protocol was stopped. One bird lost its regrowing P7, and one feather was lost during analysis, both from the food restriction group. We therefore collected two additional feathers from stored carcasses – S2 (regrown during the experiment) and S6 (grown in the wild). These were analyzed for CORT, but not included in analyses of feather quality. Feather CORT was analyzed as below. P7 feathers were also analyzed for feather quality. We recorded the length of the feather from the tip to the base of the calamus and the mass (excluding the calamus),

2.3 Plasma corticosterone radioimmunoassay

All blood samples were taken from the brachial vein in heperanized capillary tubes and were kept on ice after collection. They were centrifuged at 600g to separate plasma, which was stored at -20º C until the assay was run. We determined CORT concentrations in each plasma sample using radioimmunoassay (RIA) following (Wingfield *et al.* 1992). Samples were assayed in duplicate, and assay values corrected for individual recoveries following extraction. Intraassay variability was 3.4%; interassay variability was 17.7%. No statistical comparisons were made between samples run on different assays.

2.4 Feather corticosterone radioimmunoassay

We followed Lattin and colleagues (2011) for our feather CORT RIA protocol. In brief, steroid hormones were extracted with methanol from the feathers by sonicating and leaving in a 50º C water bath overnight. The feather particles were filtered out and methanol dried down. We resuspended the extract in assay buffer and analyzed each sample in duplicate. A standard RIA procedure was followed using Sigma C8784 antibody in the recommended Tris buffer. Feather CORT is reported in pg/mm, as recommended by Bortolotti (2008).

2.5 Statistical analyses

All statistical tests were completed in R (R Core Team 2013). Plasma CORT from the CORT in DMSO validation tests was analyzed by a repeated measures ANOVA with Treatment*Time as fixed effects and Bird as a random effect. An interaction was found, so each timepoint was analyzed independently by ANOVA followed by Tukey's post hoc analysis.

In Experiment 1, the birds lost and then regained weight, so weight change was non-linear over time. We conducted analyses on the percent of original mass. We ran a quadratic regression over time, with individual bird as a random variable (formula: Percent.weight ~ Treatment*Day^2). For the feather CORT analysis, we ran a two-way repeated measures ANOVA with day of plucking (original or regrown feathers), treatment, and their interaction. When we found a significant interaction, we then ran an ANOVA to test for differences between groups in the original feathers, then analyzed the regrown feathers in a separate ANOVA. When significance was found, we conducted a post-hoc analysis to determine difference between the treatment groups. We also ran paired t-tests to determine if CORT was different in the regrown feathers compared to the original feathers. For this analysis, each treatment group was analyzed separately.

In Experiment 2, we ran a two-way repeated measures ANOVA on bird mass (expressed as a percent of initial mass), with time and treatment as fixed effects and bird as a random effect. When a significant interaction was found, repeated measures ANOVAs for the effect of time were run on the treatment groups independently.

We used t-tests to look for differences between treatment groups at the beginning of the experiment in baseline CORT, stress-induced CORT, and strength

of negative feedback (percent decrease from stress induced 90 minutes after DEX injection). At the end of the food restriction period, we again compared baseline CORT, stress-induced CORT, and negative feedback between treatment groups. For feather CORT analysis, we used t tests to compare control to food restricted feathers for original P7, original S6, regrown P7, and regrown S2. We also used a paired t-test to compare original and regrown P7 feathers for all birds, regardless of treatment group. We did not construct a single model for feathers because they were run in two different RIAs and the different feathers may have regrown at different times in the original molt, meaning original feathers may not be comparable with each other.

We used a two-way repeated measures ANOVA on metrics of feather quality (mass and length) including day of plucking and treatment.

3 Results

3.1 Experiment 1 – exogenous CORT administration

3.1.1 DMSO validation

We first tested our exogenous CORT application. Treatment with DMSO alone did not change CORT levels compared to untreated birds. CORT in DMSO caused a temporary increase in plasma CORT compared to no treatment or DMSO alone (Fig. 1). Birds treated with CORT in DMSO had higher CORT at 30 and 60 minutes, but by 120 minutes, there was no difference between treatments (respectively: $F_{(2,9)}$=7.69, p=0.01; $F_{(2,9)}$=10.11, p=0.005; $F_{(2,9)}$=0.83, p=0.47).

3.1.2 Weight

The birds in all three groups lost weight during the first three weeks of the experiment, but they regained it by week 7 (Fig. 2). Because change in weight was nonlinear, we constructed a quadratic model with day, day^2, and treatment as fixed effects. Day did not have a significant effect, so we removed it from the model, resulting in a model with only significant explanatory variables (Treatment: $F_{(2,20)}$=3.66, p=0.04; Day^2: $F_{(1,358)}$=231.9, p<0.00001). Birds treated in the evening lost more weight than untreated birds or those treated in the morning.

3.1.3 Feather CORT:

We ran a repeated measures ANOVA including the effects of day of plucking (original or regrown) and treatment on feather CORT. Day, treatment, and their interaction were all significant (Day: $F_{(1,21)}$=35.7, p<0.00001; Treatment: $F_{(2,21)}$=5.62, p=0.01; Day*Treatment: $F_{(1,21)}$=7.6, p=0.003). Because of the significant interaction effect, we then analyzed feather CORT in the original feathers and those regrown in the lab in separate models. We first compared the three treatment groups using both right and left feathers (Fig. 3A). In the original feathers (grown in the wild), there was no difference between treatment groups ($F_{(2,21)}$=0.006, p=0.99). In the regrown feathers, there was a significant difference

between treatments ($F_{(2,21)}$=9.745, p=0.001). We ran a Tukey's post-hoc comparison on this model and found that birds treated with CORT in the morning had higher CORT than untreated birds (z=4.52, p<0.0001). Birds treated in the evening tended to have CORT levels in between morning treated birds and untreated animals, but they were not significantly different from either group (AM vs PM z=-2.24, p=0.08; PM vs control z= 2.18, p=0.09).

There was no difference in feather CORT between original feathers and regrown feathers for control birds ($F_{(1,23)}$=1.50, p=0.234). CORT was higher in regrown feathers for morning treated birds ($F_{(1,23)}$=30.72, p<0.0001). CORT was also higher in regrown feathers for evening treated birds ($F_{(1,23)}$=16.66, p=0.0005).

We then compared the feathers from the left and right wings (Fig. 3B). In the regrown feathers, there was no difference in CORT between the left side (where the CORT was applied) and the right side ($F_{(1,21)}$=0.642, p=0.432) and no side by treatment interaction ($F_{(1,21)}$=1.04, p=0.37).

3.2 Experiment 2 – Food restriction as a stressor

3.2.1 Weight

There was no effect of treatment group or day on the mass of the birds (expressed as a percent of their initial mass) over the course of the experiment (Treatment: $F_{(1,11)}$ = 1.43, p=0.3; Day: $F_{(1,63)}$=0.85, p=0.4) (Fig. 4). However, there was a significant treatment by day interaction (Group1:Day $F_{(1,63)}$=6.34, p=0.01). When the groups were analyzed separately, control animals experienced no change in weight ($F_{(1,29)}$=1.33, p=0.3). Food restricted animals, however, lost weight at the rate of about 0.09% of their starting mass per day ($F_{(1,34)}$=6.33, p=0.02).

3.2.2 Plasma CORT

A series of plasma samples was collected on day -3 or -1 and repeated on day 39 or 41 and analyzed for CORT. Before the experiment began, there were no differences in baseline or stress induced plasma CORT levels between treatment groups (respectively: t=-1.14, df=7.3, p=0.3; t=0.35, df=11, p=0.7). Control and food restricted animals had a similar percent decrease of CORT after DEX treatment (t=0.90, df=7.0, p=0.4). After feather regrowth, there was no difference in baseline or stress induced plasma CORT between control and food restricted animals (respectively: t=1.66, df=5.9, p=0.15; t=1.61, df=0.47, p=0.1), though control animals tended to have higher baseline and stress induced CORT (Fig. 5). There was no difference in negative feedback at the end of the experiment between control and food restricted animals (t=0.83, df=8.16, p=0.4), although control animals tended to have stronger negative feedback (Fig. 5).

3.2.3 Feather CORT

One of the food restricted birds lost its regrowing P7 feather partway through the experiment and one feather from the food restricted birds was lost during the assay. We therefore analyzed an additional feather (S2) that was also

regrown during the experiment and one (S6) that was not previously plucked and represents CORT accumulated when the feather was grown in the wild (Fig. 6A). There was no difference in feather CORT between control and food restricted birds in the original P7, original S6, regrown P7, or regrown S2 feathers (original P7: t=1.12, df=6.9, p=0.3; original S6: t=0.62, df=9.5, p=0.6; regrown P7: t=-0.55, df=4.3, p=0.6 ; regrown S2: t=-0.71, df=6.7, p=0.5). Regrown P7 feathers had less CORT than the original P7 feathers (t=3.03, df=15.3, p=0.01).

3.2.4 Feather Quality

The length and mass of P7 feathers (after removal of the calamus) were analyzed. There was no effect of day of plucking (original or regrown), treatment group, or their interaction on feather length in P7 feathers (Day: $F_{(1,10)}$=0.19, p=0.7; Treatment: $F_{(1,10)}$=0.04, p=0.9; Day*Treatment: $F_{(1,10)}$=2.37, p=0.2, Fig. 6B). There was a significant effect of day of plucking on the mass of P7 feathers, as well as a marginally significant effect of treatment, but no effect of the interaction between day and treatment (Day: $F_{(1,10)}$=21.09, p=0.001; Treatment: $F_{(1,10)}$=3.87, p=0.08, Day*Treatment: $F_{(1,10)}$=2.05, p=0.2, Fig. 6C). A Tukey's post-hoc analysis demonstrated that the mass of the original P7 feathers was not different between treatment groups (z=-1.33, p=0.51). Regrown feathers from food restricted birds were marginally significantly lighter than from control birds (z=-2.35, p=0.08). Regrown feathers were lighter than original feathers in the food restricted but not the control birds (respectively: z=-4.26, p=0.0001; z=-2.24, p=0.1).

4 Discussion

Feathers can provide a record of past exposure to corticosterone. Because they take several weeks to grow, feathers provide an integrativerecord of corticosterone including both stress induced and baseline levels. Many researchers are embracing feather corticosterone as a key part of the field endocrinologist's toolkit, but this technique is still new and some key validations have not yet been performed (Romero and Fairhurst in press). This study addresses some of the open questions about the analysis of feather corticosterone.

In Experiment 1, we added to a growing body of evidence that exogenously applied CORT is detectable in feathers (Fairhurst *et al.* 2013; Jenni-Eiermann *et al.* 2015; Lattin *et al.* 2011). Circulating CORT is deposited in the feather whether it is present in the morning or the evening. Feathers from birds with morning CORT application had non-significantly higher CORT than birds dosed in the evenings, the opposite of our predicted response. This is consistent with recent findings on the timing of CORT incorporation into the feather (Jenni-Eiermann *et al.* 2015). According to Jenni-Eiermann and colleagues, CORT is incorporated into the feather in the blood quill where cells are differentiating and becoming keratinized, and not in the base of the feather where cells are proliferating. Whether cells proliferate in the day or the night makes no difference: the feather matrix can incorporate CORT from any time when they are exposed to the blood supply. It is possible that the heightened nighttime metabolism in molting birds that was demonstrated by Cyr *et al.* (2008) is due to cell proliferation, not to cell

differentiation and keratinization. The birds exposed to CORT in the evening had greater weight loss than control birds or birds exposed to CORT in the morning. The additional metabolic burden of night-time cell proliferation may have been exacerbated by high CORT levels.

These data also suggest that the CORT present in the feathers represents circulating levels of the hormone, not locally produced glucocorticoids. Mammalian skin contains a complete HPA analog, with all the signaling molecules, receptors, and synthetic enzymes needed to create glucocorticoids from cholesterol (Slominski *et al.* 2007). While it is unknown whether avian skin can produce glucocorticoids, it has been shown to metabolize them (Bortolotti *et al.* 2009). Locally produced CORT could become incorporated into the growing feather and potentially interfere with interpretation of measured feather CORT levels. We tested whether locally produced CORT could be incorporated into the feather by topically applying our CORT treatments. CORT was applied along the feather tract on the left wing only. There was no difference between feather CORT in the left and right wing for either of the CORT treated groups. Consequently, feather CORT appears to reflect circulating CORT – enriching CORT locally has a negligible influence.

Many researchers are interested in linking levels of feather CORT to stressors in a bird's natural environment. However, while there is good evidence that exogenous CORT produces a detectable signal in feathers, and there is growing evidence that different wild populations or individuals have different feather CORT levels, there have been relatively few attempts to experimentally induce stress and determine that naturalistic stressors cause a change in feather CORT. One of the stressors of highest interest in the wild is nutritional stress. We therefore used a protocol of random food removal to stimulate mild chronic stress. This protocol did not result in changes in baseline and stress induced plasma CORT in this study or when used previously by Bauer and colleagues (2011). However, in European starlings and other avian species, food restriction has been shown to increase (Freeman *et al.* 1981; Lynn *et al.* 2003; Strochlic and Romero 2008) or decrease (Kitaysky *et al.* 2005) baseline and/or stress induced CORT. In our study, we found that negative feedback of the plasma CORT response tended to be less strong in food restricted birds, but there was no significant difference between groups. However, the birds were physiologically impacted by the protocol. Food restricted birds lost weight over the course of the experiment. Because they were not calorically restricted (they had ad lib. access to food for 10+ hours per day), this weight loss was probably due to psychological stress. The feather quality of the food restricted birds was also impacted. Although the length of feathers was the same in both groups, food restricted birds had marginally lighter feathers than control birds and significantly lighter feathers than their originals. Food restriction has previously been shown to impair feather quality (Strochlic and Romero 2008). However, although there was a physiological impact of the food restriction on feathers, there was no difference in feather CORT between food restricted and control groups in regrown feathers. A study in rhinocerous auklet chicks showed that with a more severe experimental food restriction, CORT was elevated both in plasma (at baseline) and in feathers (Will *et al.* 2014). However, in a study in

Caspian tern chicks raised on control or restricted diets, feather CORT was lower in food restricted birds even though baseline and stress induced plasma CORT was elevated (Patterson *et al.* 2015). This indicates that researchers will have to take care in interpreting feather CORT levels in wild birds. Some psychological stressors may result in a changed physiology that impacts feathers but does not result in higher feather CORT. Nutritional restriction or uncertainty tend to negatively impact feather quality, but they can cause increased, decreased, or no change in feather CORT. The magnitude and direction of change in feather CORT might be affected by species, age, severity of nutritional stress, or other factors. Without experimental validation of the effect of a stressor on feather CORT in a particular species, it will be difficult to draw conclusions about the meaning of a difference or lack of difference in feather CORT from birds with unknown histories.

Conclusions

The feather CORT assay first developed in 2008 (Bortolotti *et al.*) complements traditional plasma CORT measurements, but it is still not fully validated. In these experiments, we demonstrated that CORT is not incorporated into the feather differently at different times of day, but rather, it is probably deposited at any time the maturing feather matrix is exposed to the blood supply. Locally produced CORT in the skin, if it exists in birds, is also unlikely to interfere with feather CORT measurements – feather CORT most likely represents circulating CORT. However, feather CORT should not replace the other tools for studying avian physiology, as not all stressors that affect an animal's physiology will be recorded in feather CORT. A lack of difference in feather CORT between populations does not necessarily mean that they have experienced similar conditions.

Acknowledgements

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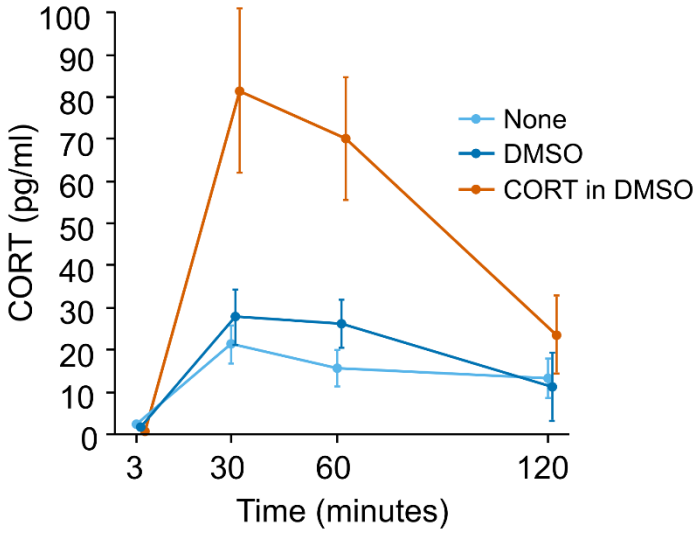

Figure 1. CORT in DMSO validation. Plasma CORT was analyzed in European starlings with no treatment, topical treatment with 20µL DMSO, or topical treatment with 20µL 1mg/mL CORT in DMSO. Plasma was analyzed at <3 minutes of disturbance, then at 30, 60, and 120 minutes after treatment. n=4 per treatment group.

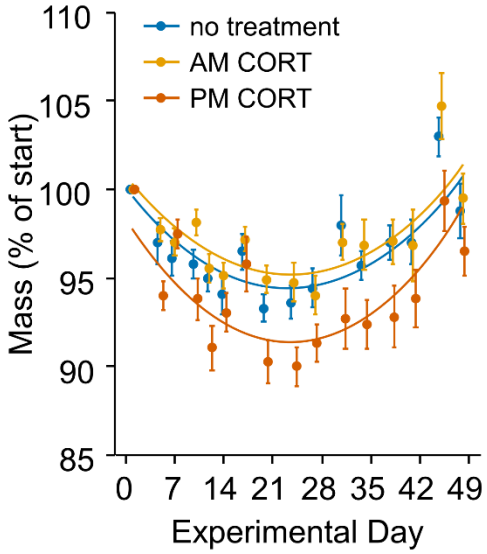

Figure 2. Change in weight during exogenous CORT application and feather regrowth. 14 flight feathers were plucked on day 0, and treatment with CORT in DMSO began on day 7. Birds (excepting the no treatment group) were treated once per day between 8 and 10 AM or between 6 and 10 PM. Data was analyzed by quadratic regression. n=8 per treatment group.

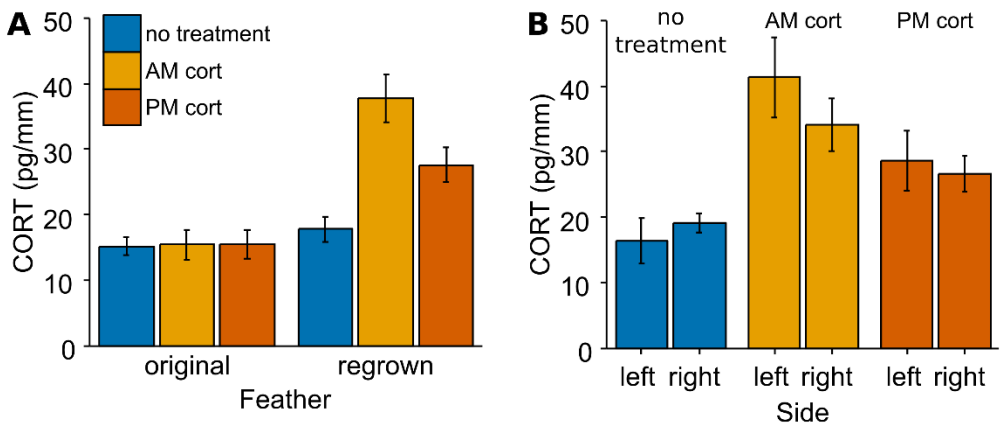

Figure 3. Feather CORT in birds with exogenous CORT treatment. S2 feather was plucked from both wings (along with 12 other flight feathers). CORT in DMSO was applied once per day starting on day 7 between the hours of 8 and 10 AM or between 6 and 10 PM. A) CORT from original S2 feathers (grown in the wild) and regrown S2. B) Comparison of left wing feathers (where topical CORT in DMSO was applied) with right wing feathers. n=8 birds per treatment group.

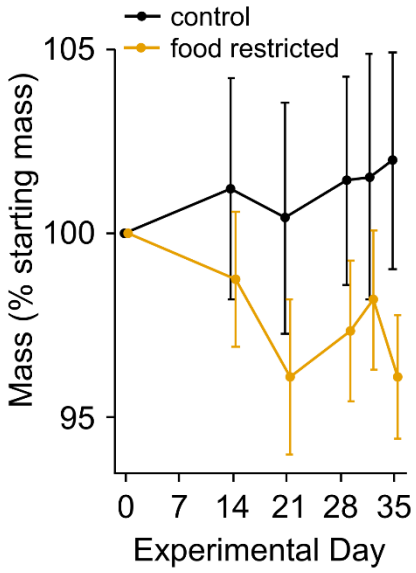

Figure 4. Mass of birds during a semi-natural chronic stressor. 12 flight feathers were plucked on day 0 to simulate molt. In the food restricted birds, food was removed for 4 hours per day at a randomly determined time point. n=6 control, 7 food restricted.

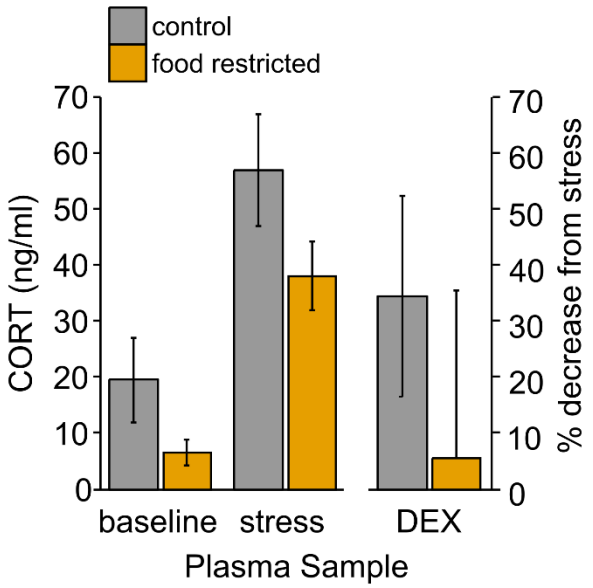

Figure 5. Plasma CORT after ~40 days of food restriction and feather regrowth. 12 flight feathers were plucked on day 0. The food restricted birds had their food dishes removed for 4 hours each day at a randomly selected time. Plasma CORT was sampled at <3 minutes of disturbance (baseline), after 30 minutes restraint in a cloth bag (stress). They were then injected with 1mg/kg dexamethasone to stimulate negative feedback (DEX; reported as the % decrease from the stress sample). n=6 control, 7 food restricted.

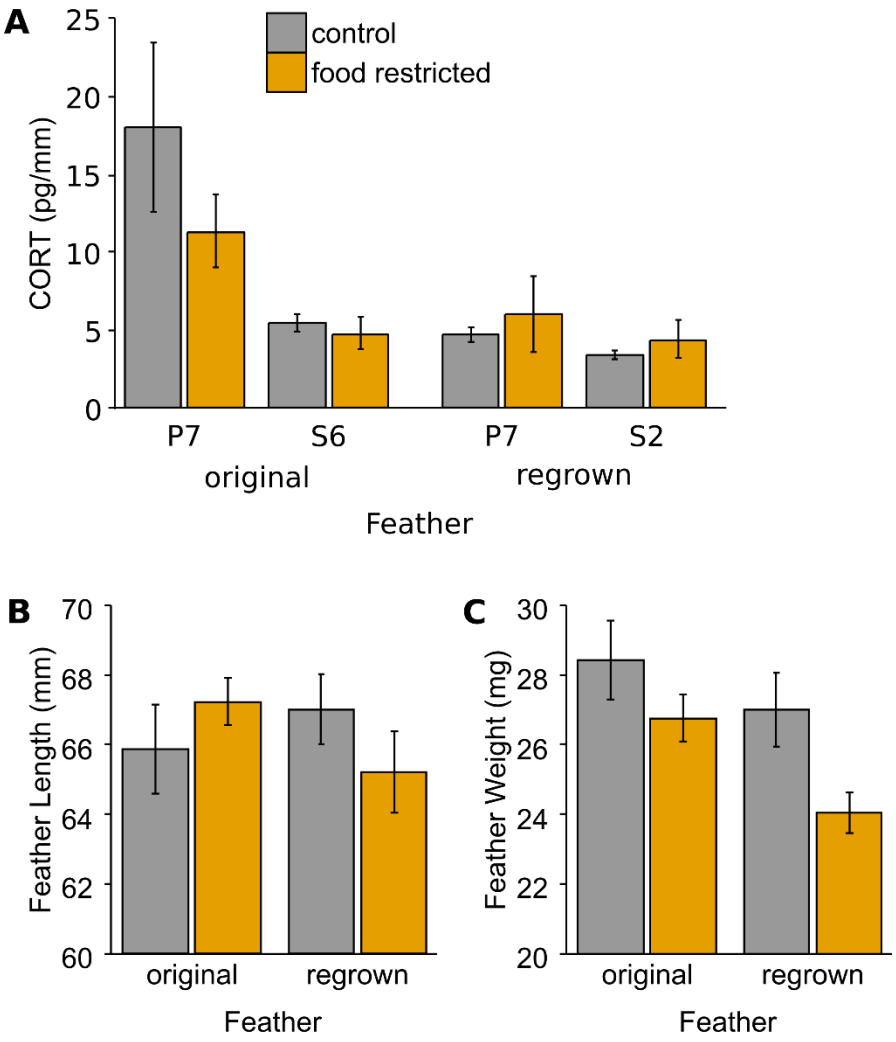

Figure 6. Feather analysis from feathers regrown during a semi-natural stressor. 12 flight feathers were plucked and regrown in the lab. Food restricted birds had food removed for 4 hours daily. A) Corticosterone in feathers grown in the wild or the lab. P7 was initially chosen as the feather for analysis, but 2 feathers from the food restricted group were lost, so an additional 2 feathers were analyzed: S6 (grown in the wild) and S2 (regrown during the simulated molt). n=6 control, 7 food restricted (except regrown P7, where n=5). B) Length (excluding the calamus) of P7 feathers grown in the wild (original) or regrown after simulated molt. n=6 control, 6 food restricted. C) Mass (excluding calamus) of original or regrown P7 feathers after simulated molt. n=6 control, 6 food restricted.

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