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DEVELOPING TOOLS TO UNDERSTAND STRESS AND NEST

ABANDONMENT IN BIRDS

By

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Thesis

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## Table of Contents

Copyright Page.....	ii
Acknowledgements.....	iii
Table of Contents.....	iv
1. Assay temperature affects corticosteroid-binding globulin and free corticosterone estimates across species .....	1
1.1. Introduction.....	1
1.2. Materials and Methods .....	5
1.3. Results.....	9
1.4. Discussion.....	10
1.5. References.....	15
1.6. Tables and Figures.....	21
2. Mechanisms of avian nest abandonment: Prolactin and corticosterone in incubating passerines	
2.1. Introduction.....	26
2.2. Methods.....	29
2.3. Results.....	35
2.4. Discussion.....	36
2.5. References.....	40
2.6. Tables and Figures.....	43

# Assay temperature affects corticosteroid-binding globulin and free corticosterone estimates across species

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## Abstract

Glucocorticoid hormones (GCs) are often measured to assess how organisms respond to challenges in their environment. In plasma, GC's circulate in two forms: bound to corticosteroid-binding globulins (CBG) or unbound (free). Measuring CBG allows us to estimate the amount of free GCs present in a plasma sample. However, free GC estimates are affected by the assay temperature used when measuring CBG, with colder temperatures maximizing specific binding but likely underestimating GC's affinity for CBG. Here, we test how a biologically relevant incubation temperature (41°C) changes the disassociation constant ( $K_d$ ; used to estimate free GC levels) when compared to the traditional 4°C incubation temperature, across four commonly studied avian species. We then apply the new  $K_d$ 's calculated at 41°C to existing data sets to examine how a change in  $K_d$  affects free CORT estimates and data interpretation.  $K_d$ 's were generally higher (lower affinity for CORT) at warmer incubation temperatures which resulted in higher levels of estimated free CORT. This increase in free CORT levels did not change previously reported patterns but did affect variance and alpha (p) values. We suggest that future assays be run at biologically relevant temperatures for more accurate estimates of free CORT levels *in vivo* and to increase the chances of detecting biological patterns that may not be revealed with the classic methodology that tend to underestimate free CORT levels.

## 1. Introduction

Across vertebrates, glucocorticoids (GCs) mediate responses to stressors and challenges to homeostasis (Sapolsky et al, 2000). The majority of field and lab studies measure total plasma GCs and seek to associate GC levels with physiological and behavioral responses (Boonstra et al, 1998; Sapolsky et al, 2000; Romero et al, 2006; Breuner et al, 2013). However, equally important are the downstream mechanisms associated with GC action such as binding globulins, receptor type/abundance and enzymatic processes (Breuner et al, 2013; Breuner et al, 2020; but see Schoech et al., 2013). Studies that focus on total hormone levels and neglect the other

components of hormone action may miss key biological relationships between endocrine mechanisms and organismal function due to their oversimplified approach. The complexity of GC physiology suggests clarification of downstream mechanisms will help us test hypotheses connecting physiological and behavioral responses that can be highly context dependent (reviewed in Harris 2020).

Corticosteroid binding globulin (also known as transcortin and hereafter CBG) regulates GC function by mediating the amount of GCs available to cells and tissues. CBG is a member of the serpin class of super proteins that binds GCs with high affinity. In vertebrate plasma, 80-95% GCs are bound to CBG (Mendel, 1989; for a mammalian exception see Desantis et al, 2013). In the comparative endocrinology field, there are two competing hypotheses regarding the action of CBG: the total hormone hypothesis and the free hormone hypothesis. Under the total hormone hypothesis, the absolute amount of GCs measured in the plasma is the biologically relevant portion, regardless of it being bound to CBG (Schoech et al 2013). Under the free hormone hypothesis, the unbound or free fraction of hormone diffuses across the vasculature to enter tissue and have biological effect (Siiteri et al. 1982, Westphal 1983, Mendel 1989, Ekins 1990). As an extension to the free hormone hypothesis, the reservoir hypothesis suggests that the bound form of CBG acts as a reservoir of GC's which can be released as needed via CBG-cleavage with neutrophil elastase (Hammond et al, 1990; Malisch and Breuner, 2010). The measurement of plasma CBG together with total hormone level allows us to estimate the amount of free hormone available to the organism (see Breuner, Beyl, and Malisch, 2020 for review), as well as the amount held in the plasma as a reservoir.

Free GC estimates can provide greater insight into the function of GC physiology in some systems by providing fine-scale information regarding GC levels. For example, in Eurasian tree

sparrows (*Passer montanus*), during a standard stress protocol, total and bound corticosterone (the primary avian GC, hereafter CORT) changed over time while free CORT was static, suggesting that these individuals may be less sensitive to environmental stressors (Li et al., 2016)). In tree lizards (*Urosaurus ornatus*), androgen-glucocorticoid binding globulins explain the decline in testosterone levels during challenge in the non-territorial morph, even if total testosterone levels are similar across the morphs (Jennings et al., 2000). Measuring CBG can also provide insight into evolutionary trends. New world flying squirrels exhibit much higher total CORT than other vertebrates but levels of CBG remain similar to phylogenetically related groups, suggesting a different mechanism underlying high total levels in this group (Desantis et al, 2018). The relevance of measuring free CORT levels remains a matter of debate (Breuner & Orchinik, 2013; Schoech et al 2013) and we recognize that free and total CORT measures do not always differ (see Patterson et al. 2014). However, in some systems, free CORT estimates elucidate associations between hormonal mechanisms and physiological/behavioral traits that would otherwise be missed if researchers only considered total hormone measurements.

Only a fraction of GC studies measure CBG levels (Breuner, Beyl, Malisch, 2020). One reason for this may have to do with the methodological concerns and difficulties of measuring CBG to estimate free CORT (Schoech et al 2013). One difficulty is that the amount of plasma required for direct measurement of free hormone may be unattainable in many small birds and mammals. Another concern focuses on the current methods used for measuring CBG that are optimized at cold or room temperatures (4°C or 26°C) in order to maximize the specific binding of CBG to GCs (necessary for calculating free CORT estimates). Body temperatures in endotherms range from ~37-41 °C and it has been argued that measuring CBG at a much colder temperature could result in a biologically irrelevant measure of CBG that is not representative of



how the protein behaves *in vivo* (as suggested in Schoech et al 2013). Indeed, in humans, affinity of CBG for CORT decreases 16-fold as temperature increases during fever from 35 - 42°C (Cameron et al., 2010). We should expect that these temperature differences occur in other species as well. Knowledge gaps around specific temperature effects and other assay conditions may be hindering widespread use and acceptance of CBG in comparative studies. Therefore, assessing how temperature affects the disassociation constant and eventually the estimates of free CORT is a major step towards resolving the concerns about the use of measures of CBG in ecological field studies.

Our aims for this study are twofold: to examine the effect of assay temperature on calculating CBG levels across several species, and to understand how assay temperature affects free hormone estimation and interpretation of results from published data sets. To address these issues, we optimized CBG assays at the typical avian body temperature (41°C) and at a standard temperature (4°C) for four bird species commonly used in lab and field studies: house sparrows (HOSP: *Passer domesticus*), European starlings (EUST), zebra finches (ZEFI: *Taeniopygia guttata*) and two white-crowned sparrow subspecies (*Zonotrichia leucophrys gambelii* and *Z. leucophrys pugetensis*). We then applied our new free CORT estimates to existing data sets on European starlings, zebra finches and white-crowned sparrows to re-evaluate the data and examine potential differences caused by a biologically relevant assay incubation temperature. Based on previous work in rodents and humans, we expect temperature to influence both the disassociation constant and free CORT estimates; however, both the magnitude of the change and how this affects the interpretation of results are unknown. This study is the first to test a biologically relevant assay temperature on free CORT estimates and apply it to field and

laboratory data. As such, it addresses concerns surrounding CBG assay parameters and suggests a way to increase their biological validity.

## **2. Materials and Methods**

### **2.1. Assay optimization at 4 vs. 41°C**

Two fundamental aspects of CBG function are affinity,  $K_d$ : the rate/strength of the binding interaction, and capacity,  $B_{max}$ : the total amount of binding globulins present. Ligand-binding assays determine the dissociation constant ( $K_d$ ) and  $B_{max}$ , which are used together with total CORT to estimate free CORT levels (Barsano and Baumann, 1989, see Hulme and Trevethick 2010, for a review of ligand binding assays at equilibrium).

#### **2.1.1 Study animals and plasma collection**

We obtained house sparrow ( $n=6$ ), European starling ( $n=4$ ), and white-crowned sparrow ( $n=5$ ), (*Z.l.gambelii*) plasma from wild-caught birds in December-February in Missoula and Florence, MT. Researchers (LM & QM) at the University of British Columbia provided us with plasma from *Z.l. pugetensis* ( $n=16$ ) as part of their ongoing study. Zebra finch ( $n=4$ ) plasma was obtained from locally purchased captive birds. For all species, samples were obtained from both sexes and pooled for use in assays. Blood samples were collected in under five minutes for all species to reduce the likelihood that handling time affected CBG capacity (as per Breuner et al., 2006). Blood was collected in heparinized capillary tubes, stored on ice, centrifuged at ~10,000 rpm within 2 hours and stored at -20°C until assayed. All animal use methods were approved by the IACUC Committee at the University of Montana (AUP 005-19CBOBE-031419) and the ACC Committee at the University of British Columbia (A17-0049).

#### **2.1.2. CBG Assays and free CORT estimations**

To measure CBG, we followed the methods of Deviche et al., (2001) with additional temperature experiments described below. Briefly, plasma was stripped of endogenous steroids through a 20-minute incubation with dextran-coated charcoal. In each assay total CORT binding was determined with 3H-CORT, while non-specific binding also included 500-1000-fold unlabeled CORT. As per standard methods, we performed the following assays associated with CBG assay optimization at both 4 and 41 °C for each species.

- 1) The plasma dilution assay tested specific binding across six plasma dilutions at both temperatures to determine a plasma dilution where less than 10% of 3H-CORT is bound, to meet the assumptions of pharmacological analyses; all subsequent assays were performed at this dilution.
- 2) The 41 °C time and temperature assay was run across 18 time points from 1 to 35 minutes. The 4 °C assay was run over 6 time points from 5 to 120 minutes. This assay determines the optimal timeframe where the binding on/off rate has reached equilibrium, but the protein has not suffered significant degradation. All following assays were incubated for either 90 minutes (4 °C) or 15-25 minutes (41 °C, dependent on species).
- 3) We ran equilibrium saturation binding curves (ESB) from 0.23 nM to 12 nM 3H-CORT at 4 °C, and from 0.5 nM to 20 nM 3H-CORT at 41 °C, to accommodate equal sampling around the respective  $K_{dS}$  at each temperature.

At assay termination, all 41 °C samples were put into an ice slurry for one-minute to slow the assay reaction in order to limit errors during separation of bound and free fractions due to rapid off-rates. 3H-CORT bound to CBG was then separated from unbound CORT by rapid vacuum filtration (Brandel Harvester) over glass fiber paper (GF/B, Brandel) that had been soaked for 1-hour in 25mM Tris plus 0.3% polyethylenimine. Filters were rinsed with approximately 500 ml

ice-cold 25mM Tris. Following filtration, filters were submerged in 5 ml scintillation fluid and vortexed; radioactivity was determined with standard liquid scintillation spectrophotometry. All samples were run in triplicate.

Free CORT concentrations were calculated using the Barsano and Baumann equation (1989) as described by Deviche et al. (2001). This equation estimates free hormone levels for binding globulin systems and is shown below in equation 1.

$$H_{free} = 0.5 \times \left[ \frac{H_{total} - B_{max} - \frac{1}{K_a} \pm \sqrt{2 \left( B_{max} - H_{total} + \frac{1}{K_a} \right) + 4 \left( \frac{H_{total}}{K_a} \right)}}{2} \right]$$

Capacity and affinity for CBG was determined by fitting untransformed data to appropriate equations using iterative, least-squares, curve fitting techniques in Prism (Graphpad 8.3.0, San Diego, CA). Optimal plasma dilution and incubation time were determined through visual assessment of data plots.

## 2.2. Re-analyses of free CORT estimates from existing datasets

### 2.2.1 Dataset information

After we obtained  $K_d$  estimates for plasma samples assayed at 4 and 41°C, we applied these new values to previously published data sets to re-estimate free CORT.

European Starlings: In Love et al. (2004), starling females were captured at night in various stages of reproduction (egg laying, incubating and chick rearing). At capture, birds were exposed to an acute stress protocol and had blood samples taken at 0- and 30-minutes. Total CORT and CBG were measured for each individual.  $K_d$  was measured at 4°C using pooled plasma samples and free CORT estimated using Barsano and Baumann (1989; this method of pooling plasma and estimating free CORT is the same for all following studies). Free CORT, but not total CORT,

was significantly higher in birds that abandoned compared to birds that did not abandon their nests after capture across reproductive stages. Data from this study are reproduced in figure 3.

Zebra Finches: In Breuner et al. (2006) ten avian species were tested to examine how CBG changed over 30- to 60-minutes when birds were faced with acute capture and handling stress. Free CORT was estimated using static CBG levels (by applying 0-minute CBG to each measure of total CORT, as is commonly done) and dynamic CBG levels (applying CBG measured at each time point). Zebra finches showed significant decline in CBG over 60-minutes of capture and handling stress, so that free CORT estimations at 30- and 60-minute timepoints were much higher when calculated with dynamic CBG levels.

White-crowned Sparrows: Breuner et al. (2006) measured how stress physiology differs in three white-crowned sparrow (*Zonotrichia leucophrys*) subspecies breeding at different latitudinal gradients. Male white-crowned sparrows breeding near Toolik, AK (*Z.l. gambelii*), Seattle, Wa (*Z.l. pugetensis*), and Sonora, CA (*Z.l. oriantha*) were captured and restrained in a cloth bag (i.e. a standard stress protocol). Birds were bled within 0-3 minutes for a baseline sample and again at 30-minutes for a stress-induced sample. Total CORT levels did not differ between populations, but free CORT levels were significantly different between *Z. pugetensis* (PSWS) and *Z. gambelii* (GWCS). We re-measured  $K_{ds}$  at 4°C and 41°C for these two subspecies.

### **2.3 Statistical analyses**

All statistics and dataset analyses were analyzed in either R (Version 4.0.2) or (Graphpad 8.4.2, San Diego, CA) by applying the original statistical methods outlined for each species to new data (2.1.2) and to datasets using new  $K_d$  estimates (2.2.1). To test for differences between  $K_d$ 's estimated at 4- and 41°C we used t-tests in Prism which allowed us to account for each

point on the curve. Starling data was analyzed using two-way analysis of variance (ANOVA) models and to examine interactions between breeding stage and nest desertion, separate ANOVAs were performed for each breeding stage (Love et al., 2004). White-crowned sparrow data was analyzed using ANOVA models for differences between *Z. pugetensis* and *Z. gambelii* (Breuner et al. 2003). Zebra finch data was analyzed by comparing the x-fold increase in CORT between the static and dynamic CBG levels for each time point and temperature and then compared statistically using a two-way ANOVA (Breuner et al. 2006).

### **3. Results**

#### **3.1. Effects of temperature on assay dynamics**

Specific binding increased until reaching equilibrium ( $K_{off}=K_{on}$ ) where it stayed until protein degradation occurred and binding decreased. All species reached equilibrium within 2 hours at 4°C and within 30 minutes at 41°C. Maximum specific binding achieved over the time course decreased at 41°C, see Fig 1A for representative house sparrow data. Optimal plasma dilution varied across species (house sparrow—1:900; zebra finch—1:1250; starling—1:720; white-crowned sparrow—1:900). Incubation temperature did not affect the final plasma dilution (Figure 1, shown for house sparrow). 3H-CORT bound to CBG with weaker affinity and lower capacity at 41°C in house sparrows (shown in Fig 1), zebra finch, starlings and the pugetensis white-crowned subspecies (Table 1). There were no temperature effects on  $K_d$  in Gambel's white-crowned sparrow (Table 1).

#### **3.2. Effects of temperature on free CORT estimations**

##### **3.2.1 European Starling**

At both incubation temperatures, free CORT was higher in the incubation and egg-laying stages in abandoning vs. non-abandoning birds. Following the methods in Love et al. (2004)

separate ANOVAs were run for each stage and repeated at both temperatures. Free baseline CORT was higher in abandoning birds compared to non-abandoning at both temperatures for incubating and chick-rearing birds but not for laying. [laying, cold:  $F_{1,33}=3.529$ ,  $p=0.069$  and hot,  $F_{1,33}=3.477$ ,  $p=0.071$ ; incubation:  $F_{1,12}=4.86$ ,  $p = 0.048$  and hot,  $F_{1,12}=4.75$ ,  $p=0.0499$ ; chick: cold,  $F_{1,6}=8.357$ ,  $p=0.027$  and hot,  $F_{1,6}=8.231$ ,  $p = 0.029$ ; fig. 3].

### **3.2.2 Zebra Finch**

Free CORT estimates were higher with the increased  $K_d$  at warmer incubation temperature for both static and dynamic ranges of CBG (Fig. 2). At 4°C, there was a 6-fold increase of static CBG in free CORT from baseline to max CORT and a 19-fold increase at 4°C with dynamic CBG. At 41°C, there was a similar 6-fold increase of free CORT when calculated using static CBG and a 16-fold increase using dynamic CBG. Separate two-way ANOVAs run in Prism Graphpad, show that free CORT calculated with dynamic and static CBG are significantly different at both incubation temperatures (cold,  $F_{1,8} = 5.887$ ,  $p=0.041$ ; hot,  $F_{1,8}= 5.763$ ,  $p=0.043$ ).

### **3.2.3 White-crowned Sparrow**

Free CORT estimates did not change for Gambel's white-crowned sparrow but did increase in the pugetensis white-crowned sparrow. At baseline levels, Free CORT remained higher in pugetensis compared to Gambel's at both hot ( $F_{1,15} = 118$ ,  $p < 0.0001$ ) and cold ( $F_{1,14}=16.07$ ,  $p=0.001$ ) incubation temperatures. Stress-induced levels show the same pattern with pugetensis levels higher than Gambel's at both incubation temperatures (hot,  $F_{1,13}=116$ ,  $p < 0.0001$ ; cold,  $F_{1,13}= 23.23$ ,  $p=0.0003$ ; Fig. 4).

## **4. Discussion**

CBG plays an important role in GC action and estimates of free hormone levels can provide researchers with a more nuanced understanding of how vertebrates respond to

environmental disturbances. Historically, the use of CBG levels in comparative endocrinology has been criticized, in part, because of assay methods that measure CBG at biologically irrelevant temperatures (4°C; Schoech et al. 2013). Here, we tested the hypothesis that incubation temperatures used in the laboratory to measure CBG levels misrepresent conditions *in vivo* and potentially affect free CORT estimates by calculating affinity ( $K_d$ ) at avian body temperature. We found assays run at 4°C underestimated free CORT in four of our five subspecies/species analyzed when compared to assays run and analyzed at the average avian body temperature (~41°C). We re-analyzed pre-existing data on each species with  $K_d$ 's calculated at 41°C and found that patterns and significance remained consistent with published results despite a change in the amount of free CORT present.

Four of our species (EUST, ZEFL, HOSP, PSWS) had a higher  $K_d$  (lower affinity for CBG to CORT) when incubated at 41°C while the Gambel's WCSP showed no significant differences between hot and cold incubation temperatures. We expected differences in the disassociation constant between species based on previously published research (see Breuner et al, 2006 for avian examples and Desantis et al 2013 and 2016 for mammals). However, no study to date has investigated how incubation temperature influences free CORT estimates in birds and how this, in turn, can affect published datasets. We re-analyzed data for each species at 4- and 41°C and found that free CORT estimates increased in all species where we also observed an increase in  $K_d$ . Interestingly, we found no response to increased incubation temperature in samples from Gambel's white-crowned sparrows. Gambel's white-crowned sparrows produce a single brood each breeding season and, as such, should show a blunted response to stress (Breuner et al. 2003). It is possible that Gambel's white-crowned sparrows have evolved mechanisms to allow them to fine tune the amount of free CORT present such as deviations in



the molecular structure or glycosylation of their CBG molecule in addition to an overall increase in CBG levels.

We expected that a 41°C incubation temperature would affect our free CORT estimates because CBG is known as a ‘protein thermocouple’ and is highly sensitive to temperature (as demonstrated in  $K_d$  data from human febrile patients; see Cameron et al., 2010). Molecularly, the structure of CBG has a reactive center loop and a binding pocket that can exist in a relaxed (R-) conformation or a stressed (S-) conformation. It is thought, as with other serpins, that when the reactive center loop moves into the molecule, it pushes out the binding pocket resulting in lower affinity for CBG to CORT; a process highly dependent on temperature (Zhou et al 2008). The sensitivity of CBG to temperature is likely why we see differences between assay incubation temperatures indicating that our assays at 4°C are not accurately capturing *in vivo* conditions.

Glycosylation (i.e. the addition of a carbohydrate to a functional group) also affects CBG affinity for CORT as temperature changes. Chan and colleagues (2013) showed that glycosylated CBG responded to increases in temperature in both the S- and R- conformations over a 5°C increase of clinically relevant temperatures. The number of glycosylated sites on the CBG molecule affects CBG’s response to temperature (Vaschenko et al 2016). In our study, the two white-crowned sparrow subspecies differed in how responsive they were to temperature. As predicted, Pugitensis’  $K_d$  increased but unexpectedly Gambel’s showed no response to incubation temperature (the lack of response was observed over several assays). It is possible that the glycosylation state of the Gambel’s WCSP CBG molecule buffers the CBG molecule to changes in temperature. Another possibility is that there is variation in the CBG amino acid sequence between the sub-species that results in different affinity capabilities at 41°C. Overall, there may be many molecular changes that affect the binding properties of CBG to CORT. However,

whether these changes produce differences that are relevant to interpretations of biological questions remains an exciting question in the field of comparative endocrinology.

While there are clear differences in CBG across temperatures in endotherms, effects of temperature change may be much greater in ectothermic and hibernating vertebrates. Generally, ectotherms have lower CORT when sampled at lower temperatures (Racic et al 2020; Anderson et al, 2017). However, if basal temperature is increasing, then we would expect the disassociation constant to also increase, resulting in increased levels of free CORT due to a temperature-induced change in affinity. Temperature effects on hormones in ectotherms have been receiving more study in light of climate change (Racic & Langkilde 2020; Dupoué et al., 2013; Telemeco & Addis 2014). But we need to develop a better understand of temperature-dependent hormonal traits (e.g. CBG activity) in order to fully understand how organisms will respond to a rapidly warming world. Glucocorticoids have broad effects on organismal function whereas CBG is more likely to fine-tune organismal responses (Ketterson & Nolan, 1999). For this reason, we may be more likely to observe changes in the structure of the CBG molecule or GC-receptor number than to GCs themselves.

Our goals of this study were two-fold: 1) to investigate how incubation temperature affect CBG assay results and interpretation; and 2) to help clear up ambiguity surrounding free CORT estimates from CBG assays. We have shown that incubation temperature changes the resulting disassociation constant used for free CORT estimation. The magnitude of this change is species specific and even within a species, geographic location seems to influence temperature induced changes in free CORT estimates. More research is necessary to understand the impact of assay temperature on the GC action across species and taxa. However, here we show for the first time that that CBG is sensitive to changes in assay temperature in commonly studied avian species

and could affect results and data interpretation if not measured properly. More studies like ours that seek to understand the mechanistic properties of CBG in systems other than human and rodent models will aid in understanding how organisms will respond to various biotic and abiotic challenges. The importance of measuring CBG and applying estimates of free CORT levels to biological patterns remains a matter of debate. Despite this, few studies have sought to directly resolve this outstanding conflict by testing the concerns related to measuring CBG. Scientists have raised testable hypotheses that will help resolve the long-standing debate about the importance of free- versus total hormone levels. Studies like ours that seek to improve the efficacy and biological relevance of the CBG assay will hopefully increase the use of free CORT in the future.

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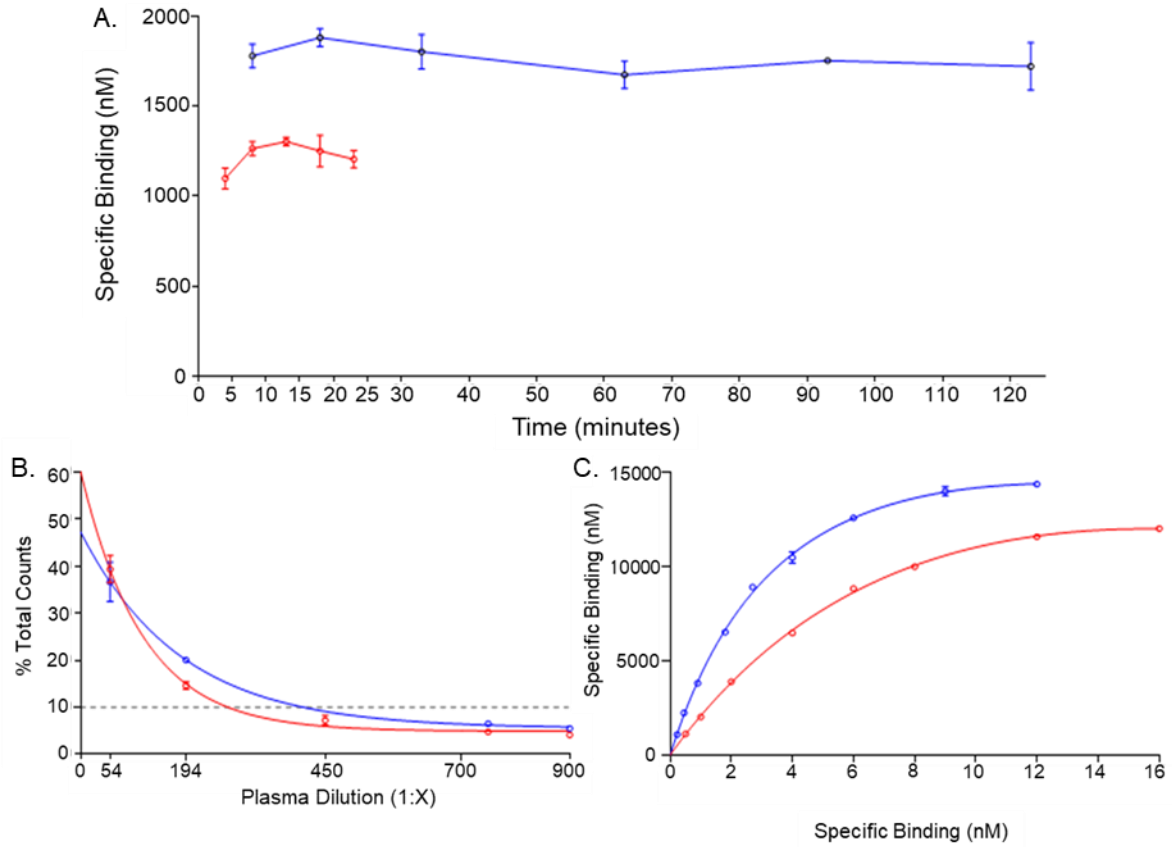
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## Tables

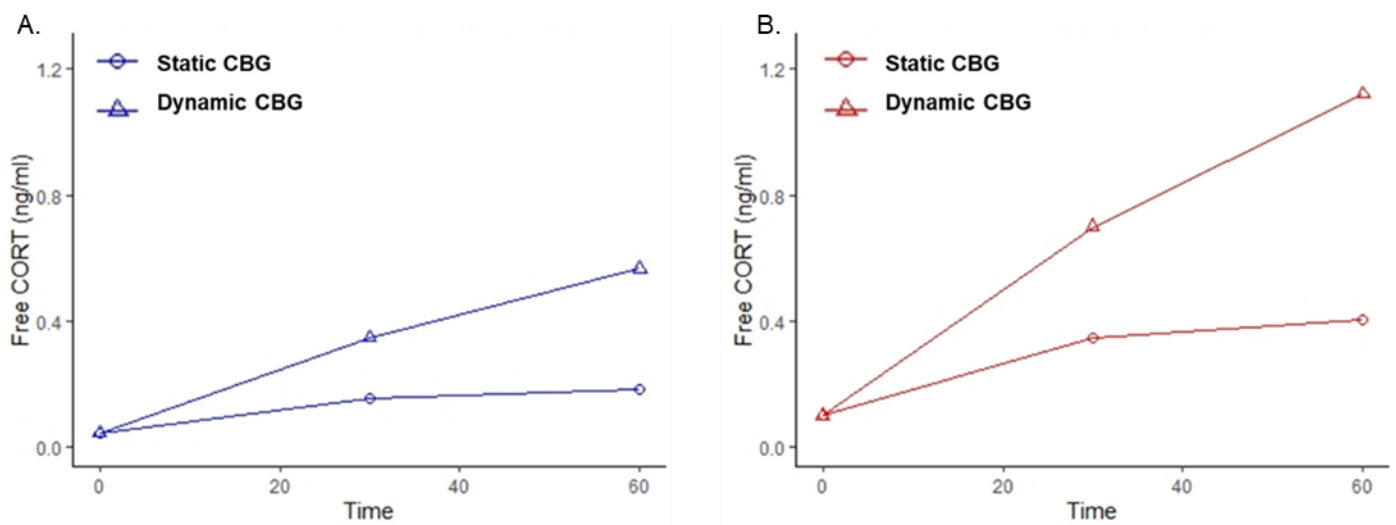
**Table 1.** Estimated disassociation constants for each species with fold change and t-tests comparing significant differences between  $K_d$  measured at 4- and 41°C

Species	$K_d$ (nM)		Fold change	Comparison of $K_d$ s	
	4°C	41°C		<i>t</i>	<i>p</i>
European Starling	5.08 ± 0.50	8.87 ± 5.56	1.75	0.678	0.508
Zebra finch	5.36 ± 1.45	12.48 ± 1.88	2.33	2.999	0.009
House Sparrow	4.84 ± 0.85	16.86 ± 5.01	3.48	2.365	0.031
Gambel's WCSP	2.70 ± 0.35	3.32 ± 0.63	1.22	0.860	4.203
Pugitensis WCSP	5.08 ± 1.87	24.22 ± 13.66	4.76	1.388	0.184

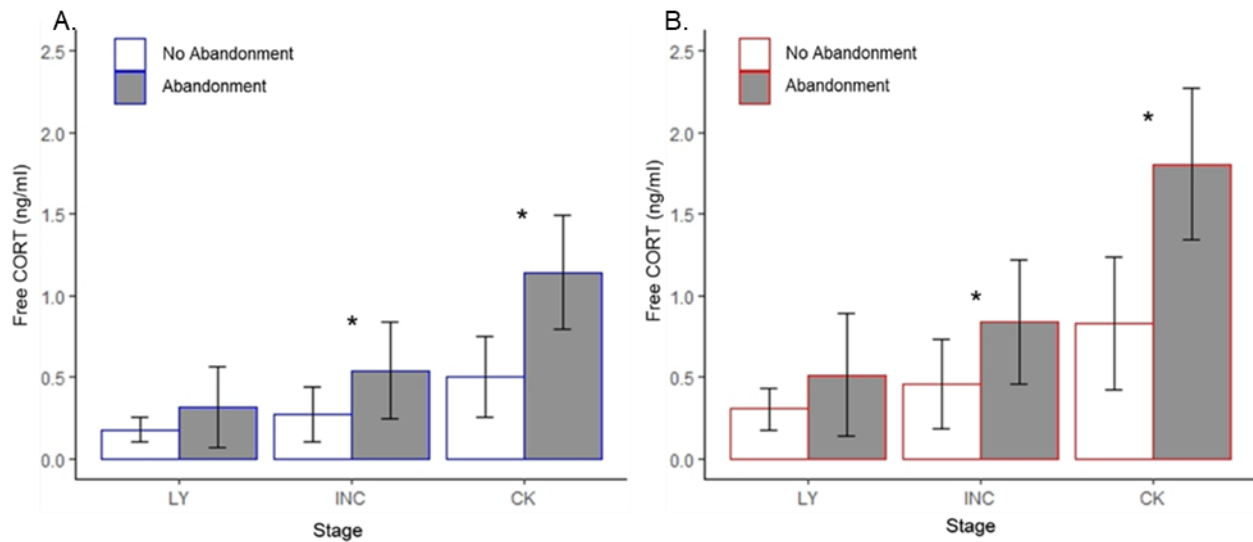
## Figures



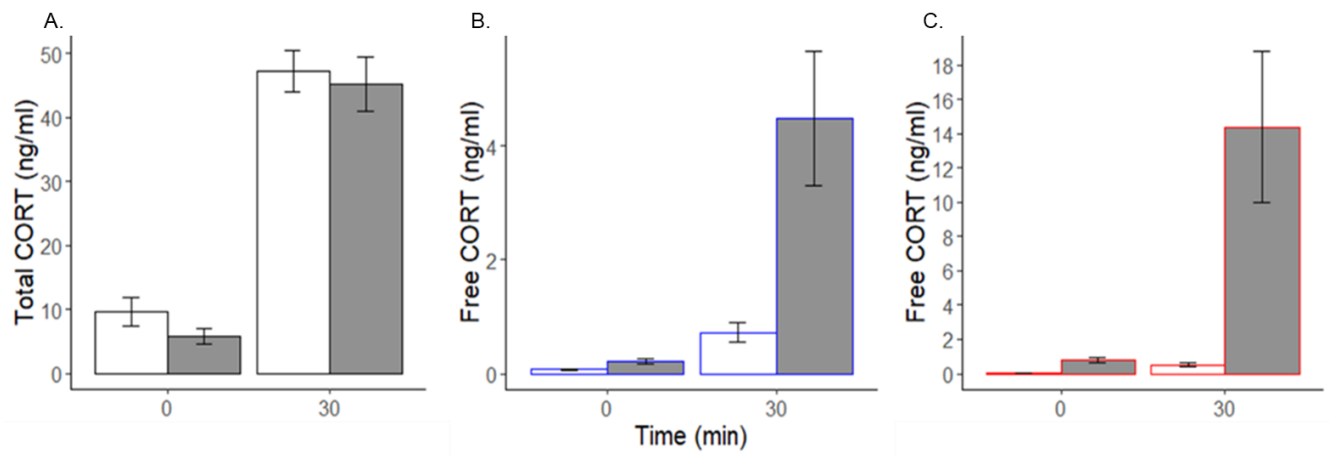
**Figure 1.** CBG validation graphs for House Sparrow pooled plasma at 4- and 41°C. A) Time and temperature at 4- (blue) and 41°C (red). B) Plasma dilution at 4 and 41. Equilibrium saturation binding curve House sparrow plotted for 4- and 41°C.



**Figure 2.** Dynamic measures of CBG increase free CORT measures over time in zebra finch plasma at both (A) 4- and (B) 41°C. Fold change remains the same for static CBG for both temperatures showing a 6-fold increase over time. Dynamic CBG increases 16-fold at cold temperatures and 19-fold at hot temperatures.



**Figure 3.** Patterns between abandoning and non-abandoning European starlings remain the same even when a warmer Kd increases overall free CORT values [laying (LY, n = 35), incubation (IN, n = 14) and chick rearing (CK, n = 8]; mean +- SEM; \*P<0.05 level of significance. Free CORT estimated from a cold incubation temperature (A, blue outline) and a hot incubation temperature (B, red outline).



**Figure 4.** Baseline and stress-induced total CORT levels (A) are the same in Gambel's WCSP (white bar) and pugetensis WCSP (gray bar) but free CORT is higher in pugitensis at both baseline and stress-induced levels. Free CORT estimated at both (B) 4- and (C) 41°C.

## **Mechanisms of avian nest abandonment: Prolactin and corticosterone in incubating passerines**

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### **Abstract**

Challenges animals experience during reproduction create trade-offs between reproduction and survival. Understanding how individuals respond to challenges could be informative for conservation decisions if responses to challenges are predictable by hormone values. Hormones provide an intermediary mechanism linking genotype to phenotype in the study of life-history traits. Corticosterone increases in response to a stressor and supports survival in response to a challenge. Prolactin typically declines in response to a stressor and supports behaviors related to reproduction. We measured prolactin and corticosterone to test the 'prolactin stress response' hypothesis in seven species of passerine. We monitored nests and captured breeding female birds during incubation and recorded nest fate (abandon, no abandon). We found no relationship between prolactin, corticosterone and abandonment in five of our seven species. Mountain bluebirds and Brewer's blackbirds show partial support for the 'prolactin stress response' hypothesis. However, a lack of pattern across species precludes the usefulness of measurements of prolactin and corticosterone as a conservation tool.

### **1. Introduction**

Trade-offs between self-maintenance and parental effort is a fundamental aspect of life-history theory, as allocation decisions during breeding have potent effects on fitness.

Mechanisms underlying trade-off decisions lie at the heart of selection on these traits, linking genotype to phenotype, as mechanisms determine available trait movement (Ketterson and Nolan, 2003). Additionally, hormone levels may predict reproductive success or abandonment propensity across vertebrates, providing useful information as environmental conditions deteriorate due to climate change or human disturbance. Despite benefits to life-history and

conservation studies, we know very little about the mechanisms that drive survival/reproduction allocation decisions during breeding.

Hormones are central regulators of physiology and behavior, integrating internal and external cues to dictate optimal behavioral decisions in response to environmental stressors (Martin et al 2011). Two hormones in particular, prolactin and corticosterone, are integral to decisions regarding parental effort and self-maintenance. Prolactin (hereafter PRL) is a peptide hormone produced in the anterior pituitary with over 300 known functions that contribute to the reproduction, metabolism, parental behavior, feeding behavior, and the modulation of the stress response (Bole-Feysot et al., 1998; Freeman et al., 2000). Generally, PRL remains low outside of breeding and peaks during critical period of parental care (Cherel et al., 1994; Groscolas et al., 2008; Angelier et al., 2007; Buntin, 2010). Prolactin levels vary across species based on life-history strategy but generally follow one of three regimes during reproduction; 1) Birds with precocial young increase PRL throughout incubation and decrease post-hatch; 2) birds with altricial young tend to show gradual increases during incubation which decline after hatch or; 3) PRL increases midway through incubation with levels remaining high throughout the post-hatch period (PRL timing outlined in Smiley 2019). PRL is strongly associated with broodiness and parental behaviors in birds and is highest in the sex responsible for the majority of parental care (Lea et al., 1981; Buntin, 1996; Buntin et al., 2008; Angelier & Chastel 2009.).

In response to a stressor, PRL has been observed to decline over both 30- and 60-minute acute stress protocols (see Angelier et al., 2016 for a review). However, individual variation in the ability of birds to modulate their PRL response to stress results in differences in parental effort (Ouyang et al., 2011; Smiley & Adkins-Regan, 2016). Prolactin research has focused on seabirds and the doves and pigeons due to their unique life-history strategies (long-lived seabirds



show relationship to PRL and crop-milk production in doves mediated by PRL). Less studied, however, is how PRL influences reproductive decisions in passerines. In house sparrows baseline prolactin levels in pre-breeding males and females positively correlate with total number of fledglings (Ouyang et al., 2011). Research in zebra finches suggests that PRL plays a role in post-hatch parental care. Following an experimental reduction in circulating PRL, zebra finches reduced or stopped post-hatch parental care (Smiley and Adkins-Regan, 2018). These studies show that there is evidence for PRL to mediate parental investment in passerines and this makes PRL is a prime candidate mediating the trade-off between reproduction and survival.

Corticosterone (hereafter CORT) is the primary glucocorticoid in birds and is a candidate in mediating survival/self-maintenance. CORT secretion is controlled by the hypothalamic-pituitary-adrenal axis under two main regimes. First, tonic inhibition and release of CORT maintain a baseline level that display 24-hour fluctuations and activate the mineralocorticoid receptor. Second, during a homeostatic imbalance (challenge/stressor) CORT is rapidly produced and released into the blood stream, activating glucocorticoid receptors and beginning a hormonal cascade to increase glucose mobilization and suppress unnecessary functions (e.g. reproduction, digestion. There is extensive evidence that CORT is a strong candidate in mediating the trade-off between reproduction and survival and high levels of CORT during avian incubation predicts abandonment across species (Ackerman & Eadie 2003; Spée et al., 2010; Ouyang et al., 2012). Together, CORT and PRL mediate opposing sides of the trade-off between reproduction and self-maintenance and by studying them together we can increase our understanding of parental allocation decisions.

Few studies to date integrate the combined responses of both CORT and PRL in determining investment trade-offs between self-maintenance and reproduction (reviewed in

Angelier et al., 2015). The prolactin stress response hypothesis proposes that the magnitude of the decline from baseline to stress-induced levels is related to a bird's current parental investment and ability to maintain parental activities in the face of a challenge (Chastel 2005; Angelier & Chastel, 2009). There is evidence in penguins that high low PRL and high CORT influence abandonment propensity but only when birds are nutritionally compromised (Spée et al. 2010). Passerines have contrasting life-histories to long-lived seabirds, but research suggests that PRL and CORT mediate reproductive decisions in this clade as well.

Here we set out to test the prolactin stress response hypothesis across seven species of incubating passerines. The goal was to investigate relationships between prolactin and corticosterone during incubation and test whether changes in these hormones predict nest abandonment. We predicted that across all species, birds with a low stress-induced prolactin levels and higher stress-induced CORT would be more likely to abandon their nesting attempt than individuals that could attenuate these responses (less decline in PRL, less increase in CORT). To tease apart possible directionality of effects across hormone systems, we experimentally manipulated prolactin levels in captive house sparrows using osmotic mini-pumps and evaluated CORT-responses. Our study is the first to measure prolactin and corticosterone in relation to nest abandonment in multiple species of wild passerines. If we can identify patterns of PRL/CORT response that predict abandonment, this could be a powerful tool in conservation physiology when managing populations at risk.

## **2. Materials and Methods**

### **2.1. Study species and site**

We measured stress-induced prolactin and corticosterone across seven species (n=118) of passerines from 2016-2019. The seven focal species for this study included two cavity nesting

species; house sparrows (*Passer domesticus*, HOSP) and mountain bluebirds (*Sialia currocoides*, MOBL) and five open-cup nesters; Brewer's blackbird (*Euphagus cyanocephalus*, BRBL), red-winged blackbird (*Agelaius phoeniceus*, RWBL), American robin (*Turdus migratorius*, AMRO), eastern kingbird (*Tyrannus tyrannus*, EAKI) and gray catbird (*Dumetella carolinensis*, GRCA). These species differ in both survival, and nesting strategies. Males and females from each species form pair bonds during the season that are maintained until nestlings fledge. Both Brewer's and red-winged blackbirds form nesting colonies where they share in the defense of the territory (Birds of the World). Females are the primary incubators with males typically spending less than 30-minutes on the nest, if they incubate at all. Any males captured were released. Mountain bluebirds were primarily studied along an established bluebird nest box trail outside of Ronan, MT on the Flathead Indian Reservation (47.478370, -114.377034). All other species were studied at the MPG Ranch, a 20,000-acre property with which promotes conservation through restoration, research and education (MPG Ranch 2020). All birds were captured at the MPG Ranch in Western Montana or along a bluebird box trail outside of Ronan, MT on the Flathead Indian Reservation (47.478370, -114.377034). In total, we monitored 341 nests across our 7 species and captured 118 individuals for hormone analyses (Table 1).

## **2.2 Nest monitoring**

Nests were found primarily using behavioral cues during nest building and egg laying. Brewer's blackbirds and red-winged blackbird's nest in colonies, and nest searching for those species involved locating a colony and systematically searching the vegetation surrounding said colony. Once located, nests (except blackbirds) were marked with green flagging tape a minimum of 6 meters away from nest. Blackbird nests were typically in a high density and close together with multiple nests often located in one tree. In order to differentiate nests, plastic

chicken leg bands (Happy Hen Treats EZ Leg Bands) were marked with a unique number and clipped directly under the nest. All nest markers were removed at the end of each breeding season. We chose green flagging to not only eliminate unnecessary attention from predators, but additionally many pollinators (hummingbirds esp.) are drawn to brightly colored flagging and we wanted to reduce our impact on non-target species. For nest box species (house sparrow and mountain bluebird), boxes at the MPG Ranch and Ronan, MT field site were monitored every 2-3 days until nest building began. Once nests were complete, we began checking every other day to record the beginning of incubation. For open cup nests, once found they were checked every 2-3 days depending on the nest stage. Nests found in an early stage of construction were given 3-days before being re-checked while complete nests were checked every other day until the first egg. When nests were found with a full clutch, eggs were candled, and the incubation stage was calculated from guides for closely related species.

### **2.3. Sample Collection**

We captured females from all species when they were approximately 80% through their prospective incubation to collect blood samples for hormone analyses. We chose to focus on females for this study because in all of our species they provide the majority of the parental care during incubation. Prospective incubation timelines were gathered from Birds of the World (formerly Birds of North America, 2017) online for each species. A pilot study in house sparrows found 100% abandonment when captures occurred at 50% and as such it was decided to catch all future species at 80%. In many passerines, it is assumed prolactin increases and peaks near hatch and we assumed the greatest interaction between prolactin and corticosterone to occur at this time. Nest box species (house sparrow and mountain bluebird) were captured using nest box flip traps. For open-cup nesters, whenever possible, we used single-cell potter traps to capture

incubating females. Traps would be placed on the nest and set. Researchers would retreat and hide in a spot where the trap was visible. When trapping in tall grass or on the ground, we would use a tall piece of grass with a “chicken band” on top, to use as a guide to know when the trap was closed. If the nest location or bird was unamenable to a potter trap, we used mist-nets set near the nests and flushed or called birds in with playback. In 2016, all house sparrow captures took place at 10 pm. House sparrows proved difficult to capture during the day and a decision to move to night capturing was made with a 100% success rate. In this year only, house sparrows were fitted with VHF transmitters using the Rapole loop method, as a pilot study to recapture after abandoning. This group was captured 50% through their incubation period and all abandoned. Due to high nest abandonment, we made the decision to capture birds at 80% of incubation for the remainder of the study.

Blood samples were collected within 3-minutes of capture. To collect blood, we punctured the brachial vein with a 26- or 30-gauge needle dependent on species and collected blood in heparinized capillary tubes. After collecting an initial sample, birds were held in opaque bags and subsequent blood samples were collected at 10-, 30-, and 60-minutes following capture. During this hour holding time, we took head-bill, tarsus and wing to the nearest 0.1mm and weight to the nearest 0.01 gram. All birds were banded with a USFW aluminum band. All house sparrows received a unique color combination in addition to a silver band. Blood was kept on ice until it was centrifuged at 10,000 rpm at the end of the field day (within 16-hr of collection) and stored at -20°C until assayed for corticosterone and prolactin.

#### **2.4. Hormone Assays**

Plasma corticosterone concentrations were assessed using an enzyme immunoassay (EIA) kit (Enzo Life Sciences). All samples from each individual were assayed on the same plate to reduce

the effect of inter-assay variation. Assays were conducted in triplicate following the manufacturer's instructions. Inter-assay variation was 8.9% and intra-assay variation was 12.75%. Plasma prolactin concentrations was determined by a heterologous radioimmunoassay (RIA) at the Centre d'Etudes Biologiques de Chizé (Cherel et al., 1994). All prolactin samples were run in duplicate. In 2018, Inter-assay variation was 9.81% and intra-assay variation was 14.07% and in 2019 inter-assay variation was 15.48% and intra-assay variation was 12.00%. The detection limit of the corticosterone assay is 27.0 pg/ml and the prolactin detection limit is 0.45 ng/ml.

## 2.5 Osmotic mini-pump methods

### 2.5.1. Subjects and experimental setup

Female house sparrows were captured from populations at a private property in Lolo, MT and at the Fort Missoula Field Research Station between 2017-2019 ( $n = 20$ ). Birds were kept on a consistent light cycle (14D, 10L) in captivity and had access to food and water *ad libitum*. We conducted a 30-minute stress protocol for each blood sampling event (shown below).



### 2.5.2. Osmotic-mini pump surgery

We used Alzet osmotic pumps (model 1007D) to administer either prolactin or sterile saline. Birds were randomly assigned to treatment groups. Pumps were filled under aseptic conditions according to the manufacturer's instructions and the pump rate set to  $\sim 0.5 \mu\text{l/h}$ . Prior

to surgery, birds were anesthetized (isoflurane gas 3-5%, maintenance 1-3%) and the surgical area cleaned with betadine and 70% ethanol. We inserted the pumps into the body cavity via an incision centered between the pubis bone and the caudal attachment of the leg. Birds were monitored post-surgery before being placed back into their cages.

## 2.6 Statistical analysis

All analyses were completed using R (version 4.0.2 Core Team 2016). We use log-transformed hormone concentrations for analyses to improve model fit and meet assumptions of normality. We ran linear models to investigate the relationship between prolactin and corticosterone values for each of our seven species in addition to estimating the Pearson's correlation coefficient. We set corticosterone as the independent variable and prolactin as the dependent variable and included capture year as a fixed term. We zero-scaled the rate of change for CORT for species with negative values and log-transformed those values to meet normality assumptions. We used separate linear models to investigate how Julian date and mass affected hormone values. Normality was assessed using the Shapiro-Wilk test. To test the efficacy of our capture-stress protocol, we used paired t-tests to look at baseline and stress-induced prolactin and corticosterone for each species.

To investigate the influence of prolactin and corticosterone on abandonment propensity, we divided each bird data set into groups based on nest fate: abandon (n=19), no abandon (n=59) and predated (n=17). We used GLMs with a binomial distribution and logit link function to test if nest fate can be predicted from our hormone measures for species with a high enough sample size for this analysis (Brewer's blackbirds, American robin, gray catbird and mountain bluebird). We also combined all species and used GLMs to investigate how CORT influences abandonment across all species. We included species as a random effect with log mass, Julian date and year as

fixed effects in these models. PRL was analyzed separately for each species because the proteic nature of prolactin and the assay conditions make comparisons across species untenable.

Additionally, we performed t-tests for each species to examine the differences in means of hormone levels between nest fates.

For the osmotic mini-pump study, we used linear-mixed effects models with a repeated measures design to investigate the effect treatment (PRL vs Control) had on baseline prolactin levels and the corticosterone stress response. In these models, hormone levels were listed as the dependent variable, treatment was listed as a fixed factor, and individual was listed as a random factor to account for repeated measurements. Relationships between hormones were investigated using Pearson's correlation with CORT as the independent variable and PRL as the dependent variable.

### **3. Results**

#### **3.1. Hormone relationships in free-living birds**

Prolactin decreased in response to the capture-restraint protocol in five of our seven species (Table 2). Baseline CORT and PRL were only correlated in mountain bluebirds ( $R = -0.47$ ,  $p = 0.025$ ). Baseline and stress-induced prolactin did not change over the season (Julian date) for any of our species. Baseline and stress-induced CORT decreased in American robins as the season progressed (baseline,  $\beta = -0.02$ ,  $F_{9.44(1,12)}$ ,  $p = 0.01$ ; stress-induced,  $\beta = -0.01$ ,  $F_{6.00(1,12)}$ ,  $p = 0.0295$ ) but showed no trend in any of our other species ( $p > 0.05$ ). The mean ( $\pm$  SD) bleed time for baseline CORT levels were  $128 \pm 66$  s; 10-min, 9 min 56 s,  $\pm 45$  s; 30-min, 29 min 22 s  $\pm 2$  min 41 s; and for PRL levels baseline,  $139 \pm 57$  s; 60-min, 59 min, 30 s  $\pm 42$  s.



### **3.2. Abandonment, prolactin and corticosterone**

We found no association between the likelihood of birds to abandon a nesting attempt and CORT or PRL levels in American robins, red-winged blackbirds, eastern kingbirds or gray catbirds (GLM;  $p > 0.05$ ). In Brewer's blackbirds the log odds of staying with a nest attempt increased when 60-minute prolactin was high ( $\chi^2 = 5.26$ ,  $n=11$ ,  $p=0.022$ ; Figure 2A) but not at baseline levels ( $\chi^2 = 0.67$ ,  $n=11$ ,  $p=0.42$ ). The log odds of abandoning also increased with higher baseline CORT ( $\chi^2 = 6.21$ ,  $n=11$ ,  $p=0.01$ ; Figure 2B) but not stress-induced values ( $\chi^2 = 2.38$ ,  $n=11$ ,  $p=0.1228$ ). Mountain bluebirds that abandoned had lower stress-induced prolactin than those that stayed ( $t=6.49$ ,  $df=17$ ,  $p<0.0001$ ). CORT did not predict abandonment across species (GLM;  $p > 0.05$ ).

### **3.3 Osmotic mini-pumps**

Treatment did not affect hormone levels of prolactin or corticosterone. Since hormone values did not vary by treatment group, we combined them to look at the relationships between hormones. Prolactin did not predict CORT levels at baseline, 10-minute or stress-induced levels (Table 3, figure 3) but sampling day influenced 10-minute and 30-minute CORT levels (Table 3, Fig. 4).

## **4. Discussion**

Animals face tradeoffs in the relative amount of energy they allocate to parental behavior compared to survival. Understanding the hormonal mechanisms that modulate such life history tradeoffs is crucial to understanding how selection drives life-history strategies. Here, we investigated how interactions between the prolactin and corticosterone stress responses affect nest abandonment decisions in wild birds. We predicted that in all species, birds with high CORT and low PRL would abandon more readily than birds with low CORT and high PRL. In

two species, we found that birds with higher prolactin and lower CORT levels were less likely to abandon a nesting attempt compared to females with low prolactin and high CORT levels. However, this pattern was not broadly true across species. We found evidence for covariation in PRL and CORT levels in free-living mountain bluebirds but not in any of the other species tested. Overall, our results offer mild support for the prolactin stress response hypothesis but also suggest that there is greater variation in factors driving abandonment decisions than found in seabirds (species that drove the formulation of the prolactin stress response).

In house sparrows and gray catbirds, PRL levels did not change in response to our acute stress protocol. This mimics a recent study on white-crowned sparrows, which showed no change in PRL levels over a 30-minute capture-handling stress (Krause 2018). Our capture handling stress was 60-minutes specifically to capture the response of PRL to stress, which has been shown to slowly decrease in seabirds (Angelier et al., 2016). It is possible that both house sparrows and catbirds attenuate their prolactin response to stress and that is why we did not see a change. An attenuated prolactin stress response would be adaptive if it resulted in increased fitness (Chastel et al., 2005). However, we did not see relationships between abandonment and hormone levels in these species. CORT levels in American robins decreased over the course of the season. Decreasing the response to a stressor would be adaptive if it resulted in less abandonment. But, again, we saw no relationship to abandonment and Julian date in the American robin. The modulation of the stress responses in robins, catbirds and house sparrows did not relate to abandonment propensity in our study.

The mechanistic link between prolactin and corticosterone is not fully understood in birds. It is possible and likely that this link is not direct and that intermediary mechanisms control this relationship (Angelier & Chastel, 2009). In mammals, it has been shown that

pregnant mice and rats have a blunted stress-response when circulating prolactin levels are high suggesting an interaction between the two hormones (Tillbrook & Clark, 2006). We attempted to investigate this link in captive house sparrows by increasing prolactin over the course of 7-days and measuring the resulting corticosterone levels. Unfortunately, our prolactin treatment did not reliably increase PRL. This could be due to a few possibilities. Between our pilot and final study our source of ovine-PRL changed due to a lack of supply. Differences in PRL preparation could have resulted in a less effective strain. Further study into the mechanisms that could mediate prolactin and corticosterone during reproduction are needed to better understand the potential relationships between prolactin and corticosterone (reviewed in Smiley 2019). While laboratory studies can be incredibly helpful, not all species can be easily kept and measured in a lab setting. That combined with the variation in hormone responses we measured across species shows us that it is vital to test these relationships in the field when possible.

We found that both stress-induced PRL and baseline CORT predicted abandonment in Brewer's blackbirds offering partial support to our hypothesis. Birds with high stress-induced prolactin levels and low baseline CORT levels had higher odds of completing their nesting attempt (Fig. 2). Further supporting this hypothesis, mountain bluebirds that abandoned their nest had lower stress-induced prolactin values than birds that stayed. Additionally, in mountain bluebirds baseline CORT was negatively associated with baseline prolactin suggesting a potential mechanistic link between the two hormones. Mountain bluebirds have the longest nestling phase out of the birds in our study with young fledgling around 18 days. Prolactin values pre-breeding and during the incubation phase have links to care post-hatch (Smiley & Adkins-Regan, 2018, Ouyang et al., 2011). Therefore, it is possible that mountain bluebirds show an

attenuated prolactin response to stress both to deal with stressors during incubation and to maintain parental care in a long nestling phase.

Previous work in house sparrows shows a strong relationship between pre-breeding values of PRL with total number of fledglings (Ouyang et al., 2011). During incubation, however, PRL does not seem to mediate abandonment decisions in our population. Notably, only 20% of our house sparrows hatched with the rest abandoning post-capture. In American robins, gray catbirds, red-winged blackbirds and eastern kingbirds we saw no signal of CORT or PRL on abandonment despite patterns found in the Brewer's blackbird which had similar abandonment propensity as the robins and catbirds. The ability to predict abandonment propensity to stressors in species of concern could aid conservation assessments. The lack of a pattern between abandonment and hormone values in five of our seven species does not support the use of PRL and CORT as a conservation tool.

CORT and PRL are potential mediators of life-history decisions, but studies examining the specific effects of these hormones on wild passerines are limited. Here, we show that baseline CORT and stress-induced PRL predicts abandonment in the Brewer's blackbird and that abandoning mountain bluebirds have lower stress-induced prolactin than birds that do not abandon. We found no relationships between baseline or stress-induced prolactin or corticosterone in our other five species. We acknowledge that our sample sizes are low, but power tests do not suggest higher sample sizes would lead to another conclusion. We suggest that future studies experimentally manipulate hormone levels in free living birds to further test the prolactin stress response hypothesis and the interactions of PRL and CORT in mediating life history decisions.

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## Tables

**Table 1:** Mean ( $\pm$  SEM) raw hormone levels (ng/ml) for all birds sampled during study including number of birds from each species and their resulting nest fate.

Species	American robin	Brewer's blackbird	Eastern kingbird	Gray catbird	House sparrow	Mountain bluebird	Red-winged blackbird
BL PRL	89 $\pm$ 8.5	106 $\pm$ 12	142 $\pm$ 9.2	71 $\pm$ 10.9	11 $\pm$ 2.8	152 $\pm$ 14.9	83 $\pm$ 10.4
SI PRL	50 $\pm$ 2.6	58 $\pm$ 4.24	63 $\pm$ 6.1	44 $\pm$ 6.9	8 $\pm$ 2.2	43 $\pm$ 5.6	46 $\pm$ 3.9
BL CORT	7.51 $\pm$ 2.2	4.56 $\pm$ 0.6	5.92 $\pm$ 1.1	4.34 $\pm$ 0.6	1.74 $\pm$ 0.4	4.58 $\pm$ 0.6	3.27 $\pm$ 0.5
SI CORT	34 $\pm$ 4.7	26 $\pm$ 3.9	60 $\pm$ 7.2	36 $\pm$ 5.1	22 $\pm$ 1.9	24 $\pm$ 2.5	23 $\pm$ 2.9
<b>Nest Fate</b>							
Stay	8	5	11	6	2	24	9
Abandon	5	6	1	5	7	4	2
Predated	4	6	0	0	1	1	6
Unknown	1	1	1	0	0	2	0
<b>Total Nests</b>	<b>18</b>	<b>18</b>	<b>13</b>	<b>11</b>	<b>10</b>	<b>31</b>	<b>17</b>



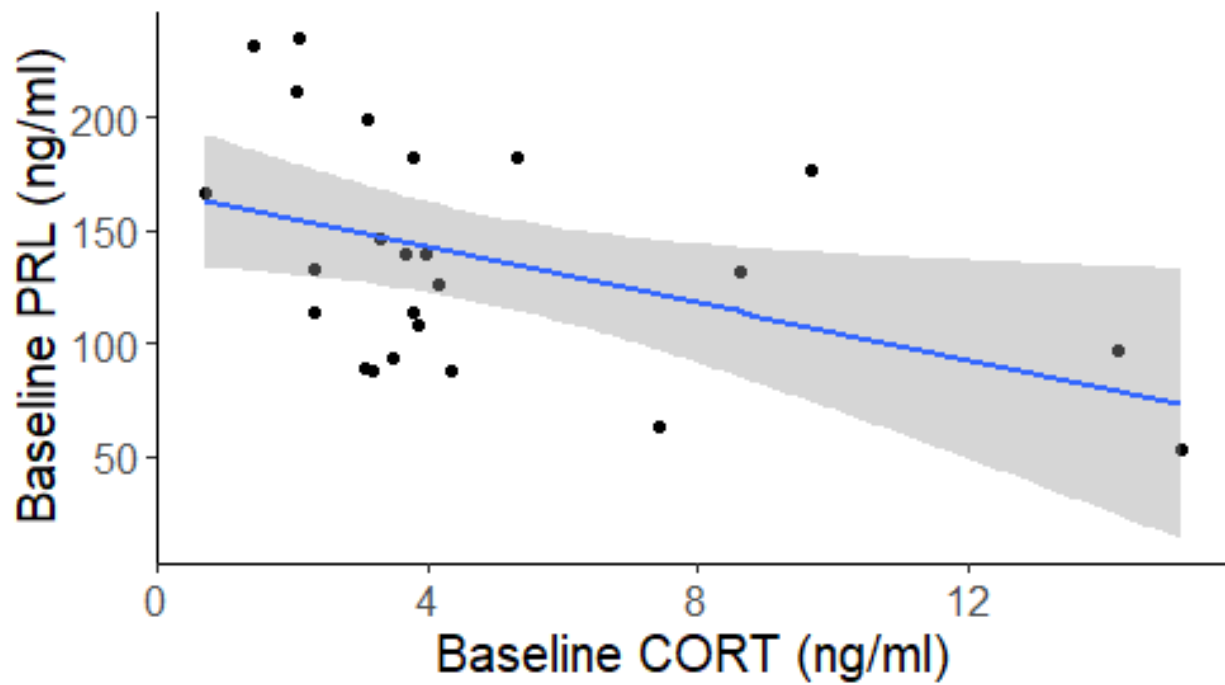
**Table 2:** Paired t-tests evaluating the magnitude of the prolactin acute stress response. Comparison of means for each species between baseline and stress-induced levels of prolactin. Table contains t, degrees of freedom (df) and p-values.

Species	<i>t</i>	df	<i>p</i>
American Robin	6.235	13	< <b>0.0001</b>
Brewer's Blackbird	4.661	15	< <b>0.001</b>
Eastern Kingbird	9.410	11	< <b>0.0001</b>
Gray Catbird	2.001	8	0.0804
House Sparrow	1.503	5	0.1931
Mountain Bluebird	7.800	23	< <b>0.0001</b>
Red-winged Blackbird	6.156	13	< <b>0.0001</b>

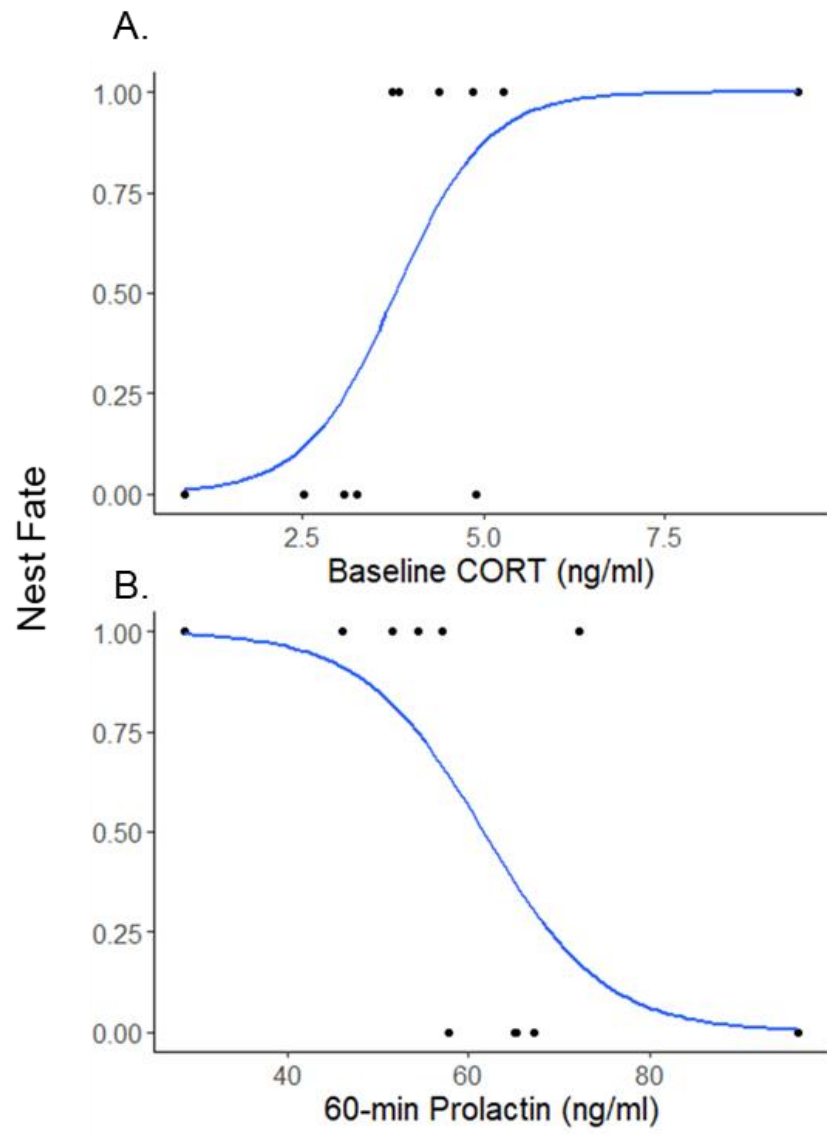
**Table 3:** Generalized linear mixed models investigating the influence of baseline prolactin (PRL) and bleed day on corticosterone (CORT) levels.

<i>Predictors</i>	Log Baseline CORT			Log 10-minute CORT			Log Stress-induced CORT		
	$\beta$	<i>CI</i>	<i>p</i>	$\beta$	<i>CI</i>	<i>p</i>	$\beta$	<i>CI</i>	<i>p</i>
Log Baseline PRL	-0.33	-0.93 – 0.26	0.273	0.08	-1.01 – 1.16	0.892	-0.40	-1.18 – 0.39	0.322
Bleed day: 2	8.30	-1.45 – 18.05	0.095	17.94	3.21 – 32.68	<b>0.017</b>	-7.48	-17.87 – 2.90	0.158
Bleed day: 4	3.42	-5.51 – 12.34	0.453	14.90	1.74 – 28.07	<b>0.027</b>	-8.27	-17.53 – 0.98	0.080
Bleed day: 7	3.09	-6.28 – 12.45	0.518	17.19	3.35 – 31.04	<b>0.015</b>	-15.37	-25.10 – -5.64	<b>0.002</b>
<b>Random Effects</b>									
$\sigma^2$	102.59			209.71			102.73		
N	14 <small>bird.id</small>			14 <small>bird.id</small>			14 <small>bird.id</small>		
Observations	37			37			37		
Marginal R <sup>2</sup> /	0.090			0.158			0.192		

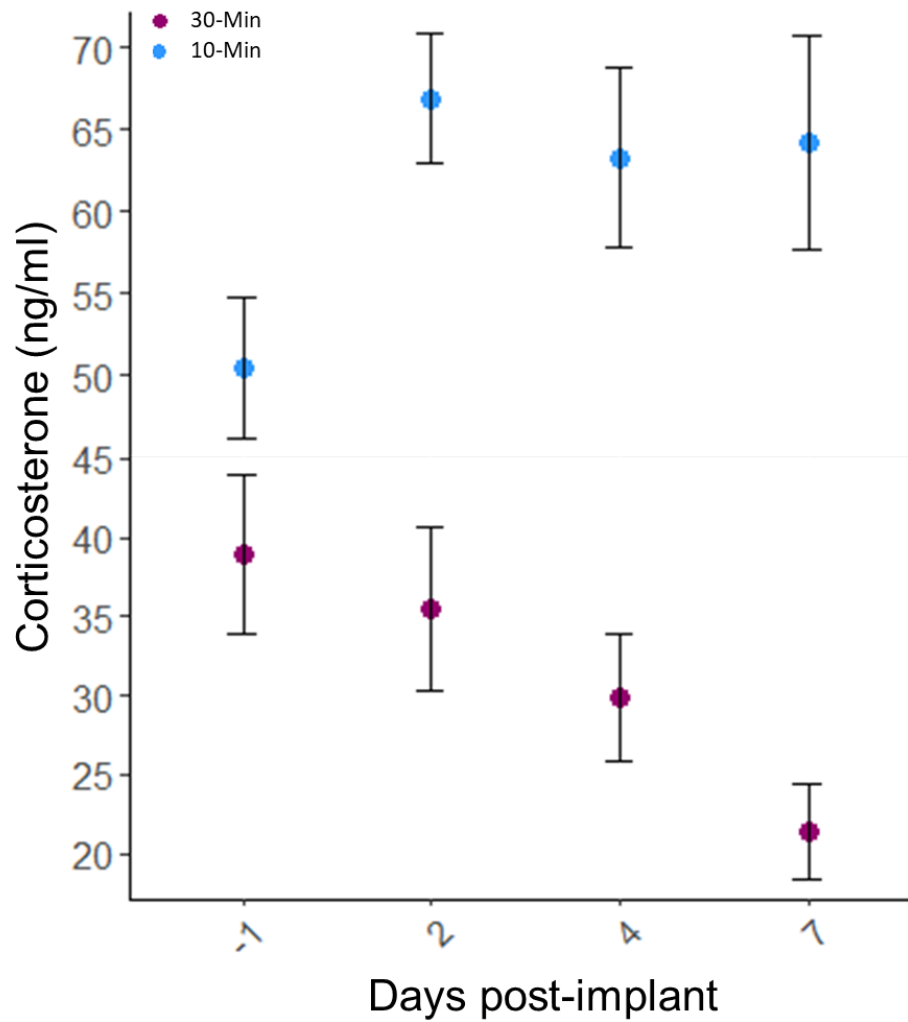
## Figures



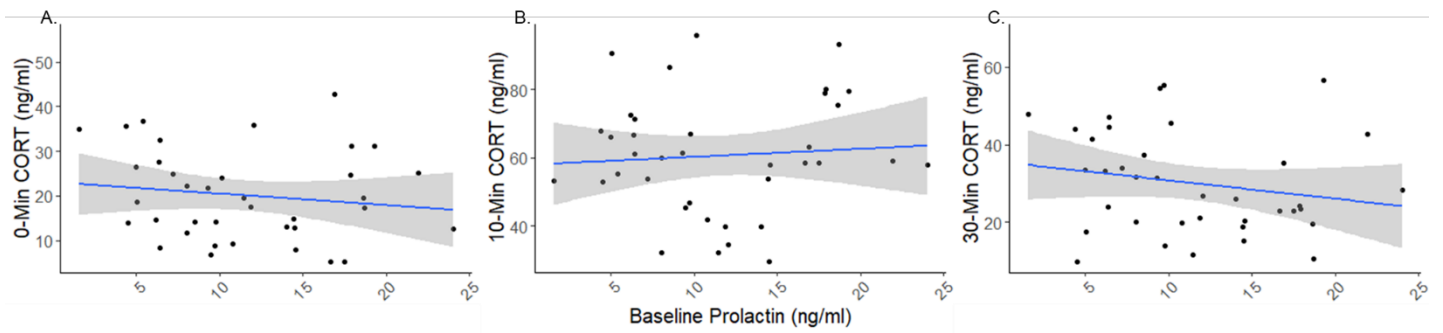
**Figure 1:** Prolactin (PRL) and corticosterone (CORT) are correlated (Pearson's  $R = -.45$ ,  $p=0.027$ ) at baseline levels in the Mountain bluebird.



**Figure 2:** Higher baseline CORT and lower 60-minute prolactin levels predict abandonment in Brewer's blackbirds.



**Figure 3:** 10-minute CORT levels (blue circles) increase over the course of the experiment and 30-minute CORT levels (purple circles) decreased in captive house sparrows.



**Figure 4:** Prolactin values did not influence corticosterone at baseline (A), 10-minutes (B) or 30-minutes (C) post capture-handling stress in captive house sparrows.