Patterns of stress response to foreign eggs by a rejecter host of an obligate avian brood parasite

Mikus Abolins-Abols[1], Mark Peterson[2], Brett Studer[3], Mattison Hale[1], Daniel Hanley[4], George Bentley[5], and Mark Hauber[3]

[1]University of Louisville
[2]Life-Science Innovations
[3]University of Illinois at Urbana-Champaign
[4]George Mason University
[5]University of California Berkeley

May 27, 2022

Abstract

One of the most effective defenses of avian hosts against obligate brood parasites is the ejection of parasitic eggs from the nests. Despite the clear fitness benefits of this behavior, individuals within so-called "egg rejecter" host species still show substantial variation in their propensity to eliminate foreign eggs from the nest. We argue that this variation can be further understood by studying the physiological mechanisms of host responses to brood parasitic egg stimuli: independent lines of research increasingly support the hypothesis that stress-related physiological response to parasitic eggs may trigger egg rejection. The "stress-mediated egg rejection" hypothesis requires that hosts activate the stress-response when responding to parasitic eggs. We tested this prediction by experimentally parasitizing incubating American robins Turdus migratorius, an egg rejecter host to obligate brood parasitic brown-headed cowbirds Molothrus ater, with mimetic or non-mimetic model eggs. To assess the stress response, we measured the heart rate in incubating females immediately after experimental parasitism. We also measured plasma corticosterone and, in a subset of birds, used RNA-sequencing to analyze the expression of proopiomelanocortin (POMC), a precursor of adrenocorticotropic hormone (ACTH), two hours after experimental parasitism. We found that egg type had no effect on heart rate. Two hours following experimental parasitism, plasma corticosterone did not differ between the differently colored model egg treatments or between rejecter and accepter females within the non-mimetic treatment. However, females exposed to non-mimetic eggs showed an upregulation of POMC gene expression in the pituitary compared to females treated with mimetic eggs. Our findings suggest that parasitic eggs may activate the stress-related hypothalamic-pituitary-adrenal axis in an egg-rejecter host species, although the dynamics of this response are not yet understood.

Introduction

Obligate avian brood parasites lay their eggs in the nests of heterospecific hosts, imposing substantial fitness costs: parasitized host broods typically experience a lower hatching rate (Hauber 2003a), higher host chick mortality (Hauber 2003b), and/or delay in the foster parents' future reproduction (Mark & Rubenstein 2013). In turn, many host species have evolved behavioral defenses against parasitic eggs. Among the most effective and common host defenses is the rejection of the foreign egg from the nest before it hatches (Rothstein 1982; Davies & Brooke 1988; Moksnes *et al.* 1991). While we know much about the behavioral and sensory ecology and evolution of egg rejection (Davies & Brooke 1988; Feeney *et al.* 2014; Soler 2014, 2017), the mechanistic basis of this host defense is still poorly understood (Abolins-Abols & Hauber 2018; Ruiz-Raya 2021). A mechanistic understanding of a phenotype can offer a unique perspective into the evolution and ecology of organisms (Ketterson *et al.* 2009; Rosvall 2013). In particular, understanding the physiological basis of

egg rejection may explain one of the most puzzling observations in host-parasite ecology, which is why the propensity to reject parasitic eggs is highly variable both among species (Stokke *et al.* 2005; Krüger 2007) and between individuals of the same species (Luro & Hauber 2017).

While variation in egg rejection across species may be explained by phylogenetic inertia and other evolutionary constraints (Rothstein 1975; Medina & Langmore 2016), most of these hypotheses cannot predict why the likelihood of egg rejection varies between populations (Davies & Brooke 1989; Soler & Møller 1990; Briskie *et al.* 1992), among individuals (Grim *et al.* 2014; Hauber *et al.* 2020a, 2020c) and within individuals over time (Ruiz-Raya and Soler, 2017, 2020). Intraspecific variation in egg rejection is particularly puzzling because this behavior has an obvious fitness benefit and typically incurs comparatively low costs (e.g., the possible rejection of own eggs (Lotem *et al.* 1995; Ruiz-Raya & Soler 2017)). Some of the intraspecific variation in egg rejection can be explained by shifts in the sensory-perceptual environment of the nest (Honza *et al.*2011; Rutledge *et al.* 2021), detecting of an adult brood parasite adult near the nest (Davies & Brooke 1988; Moksnes & Røskaft 1989), or previous experience with parasitic eggs (Hauber *et al.* 2006). Variation in egg rejection may also be linked to the host life-history stage (Ruiz-Raya & Soler 2017; Zhang *et al.* 2021). Many of these hypotheses treat egg rejection in isolation from the rest of the phenotype. However, components of egg rejection behavior may share underlying endocrine mechanisms with related behaviors (e.g., aggression, maternal behavior, nest defense (Abolins-Abols & Hauber, 2018)), suggesting that we need to study egg rejection within the rich context of other physiological and behavioral responses of the individual to its environment (Ruiz-Raya & Soler 2020; Ruiz-Raya 2021).

One of the recently emerged hypotheses about the mechanistic basis of egg rejection suggests that it is mediated by the endocrine stress response (Abolins-Abols & Hauber 2018; Ruiz-Raya *et al.* 2018; Abolins-Abols & Hauber 2020a). For example, Ruiz-Raya et al. (2018) showed that parasitism with non-mimetic model eggs elevated baseline corticosterone levels in European blackbird (*Turdus merula*) females. By experimentally suppressing glucocorticoid synthesis, Abolins-Abols and Hauber (2020a) demonstrated that corticosterone also mediates egg rejection in the congeneric American robin (*T. migratorius*). Specifically, female robins with suppressed glucocorticoid synthesis were less likely to reject non-mimetic model eggs. Combined, these findings imply that brood parasitic egg stimuli may induce a stress response which then modulates the egg rejection decisions and behaviors. Alternatively, the stress response may prime the host physiologically and cognitively to recognize and reject the foreign egg, instead of directly inducing the egg rejection response.

The emerging findings that steroid hormones play a role in response to parasitic eggs in hosts (see also Hahn et al., 2017; Hauber et al., 2020a) is important not only from a mechanistic perspective but also from an evolutionary one. Glucocorticoids are hormones that impact multiple aspects of the phenotype, including stress response, metabolism, and immune response (Williams 2008; MacDougall-Shackleton*et al.* 2019). Selection on any of these or other glucocorticoid-mediated aspects of the phenotype may cause population- or individual-level variation in glucocorticoid levels (Ketterson & Nolan 1999), thus also driving variation in egg rejection. In turn, the glucocorticoid-mediated response to brood-parasitic stimuli may drive variation in other aspects of the phenotype. For example, stress-induced corticosterone secretion can suppress maternal investment (Angelier & Chastel 2009; Angelier *et al.* 2009). This suggests that glucocorticoid-mediated responses to parasitic stimuli may induce a critical trade-off between host defenses and maternal behavior, as suggested by Abolins-Abols & Hauber (2018).

While the "stress-mediated egg rejection" hypothesis has been supported by a number of studies (Ruiz-Raya *et al.* 2018; Abolins-Abols & Hauber 2020a; Ruiz-Raya *et al.* 2021), its role in driving individual variation in egg rejection is yet to be fully understood. For example, experimental suppression of glucocorticoid results in a lower propensity for egg rejection (Abolins-Abols & Hauber 2020a); however, contrary to the predictions of the stress-mediated egg rejection hypothesis, natural circulating corticosterone in the same species shows a negative association between baseline corticosterone and the propensity for egg rejection (Abolins-Abols & Hauber 2020b). To understand if stress response can drive egg rejection decisions in diverse contexts and across varied timeframes, we need to understand the multifaceted physiological responses of hosts to parasitic

eggs.

For example, response to stressful stimuli is considerably more complex than a simple read-out of corticosterone levels. Corticosterone is regulated by adrenocorticotropic hormone (ACTH) secreted from the anterior pituitary (Romero & Butler 2007), where it is encoded by the proopiomelanocortin (POMC) gene. The POMC peptide is post-translationally cleaved to produce a variety of signaling peptides, including ACTH. ACTH secretion from the anterior pituitary is regulated by hypothalamic hormones, such as corticotropin releasing hormone (CRH) and arginine vasotocin (Romero 2006). The stimulation of the pituitary by CRH results in a rapid release of secretory vesicles containing ACTH (Deng *et al.* 2015; Harno *et al.* 2018), which, in turn, stimulates rapid glucocorticoid secretion from the adrenal cortex (Romero & Butler 2007). While it is clear that glucocorticoids play an important role in regulation behavior and physiology, it is important to note that other components of the hypothalamic-pituitary-adrenal (HPA) axis may have glucocorticoid-independent extra-adrenal effects on behavior (e.g. ACTH: Brain and Evans, 1977; Gallo-Payet, 2016; Miller and Ogawa, 1962). Furthermore, the activation of the HPA axis is only one aspects of a multifaceted response to stressful stimuli. Immediate response to stressors is enabled by catecholamine release (the fight-or-flight response), which is regulated by the sympathetic and parasympathetic branches of the autonomic nervous system (Romero & Gormally 2019). Among effects on behavior, catecholamine release results in an elevated heart rate (Cyr *et al.* 2009). Importantly, while heart rate increases in response to stressors, heart rate may be lowered during orienteering response (Hauber *et al.* 2002).

Here we tested the hypothesis that parasitic egg stimuli induce a stress response in an egg-rejecter host, the American robin. Robins are parasitized by a generalist brood-parasite, the brown-headed cowbird (*Molothrus ater*). While most robins (>90%) reject natural or model cowbird eggs (Rothstein 1982; Luro *et al.* 2018), some individuals accept natural cowbird eggs and hatch parasitic offspring (pers. obs.). Furthermore, variation in egg rejection is repeatable in robins (Luro & Hauber 2017), making this species a great model system in which to test the mechanisms underlying variation in egg rejection. We experimentally parasitized robin females with mimetic (robin-like shell color) or non-mimetic (cowbird like) eggs (Fig. 1) and measured the three aspects of host physiology in response to the model eggs: heart rate, corticosterone levels, and POMC expression in the pituitary. We predicted that, compared to host females receiving a mimetic egg, females exposed to the parasite-like model egg color treatment would show lower heart rates (i.e., orienting response), greater circulating corticosterone, and higher POMC expression.

Materials & Methods

We studied American robin females breeding in a tree farm near Urbana, IL, USA (lat: 40.128184; long: -88.105349) from April-July in 2018 and 2019. We focused only on female robins as it is the sex responsible for egg rejections in this species (Hauber et al. 2019). We surveyed the area every 3 days to detect incubating females. Incubation stage was unknown for most subjects and is not included in the analyses as it does not influence egg rejection rate in this species and is not associated with variation in corticosterone levels at early incubation stages in our study population (Abolins-Abols & Hauber 2020b).

Experimental parasitism treatment

To assess whether parasitic eggs induce a stress response in American robin females, we experimentally parasitized their nests during incubation with either a mimetic or non-mimetic model egg (Fig. 1). We used 3D printed white nylon eggs, sourced from Shapeways.com (model ID "cow bird"), which were modeled after a natural cowbird egg and are similar in shape, size, and weight to it (Igic *et al.* 2015). These eggs were painted either immaculate light blue (mimetic treatment, resembling natural robin eggshell's color) or immaculate beige (non-mimetic treatment, resembling the cowbird eggshell's color) following published protocols (Canniff *et al.* 2018; Hauber*et al.* 2019). We placed one model egg in the nest of the incubating female, without removing any host eggs: prior studies showed that the removal of own eggs in *Turdus* thrushes did not affect their responses to experimental parasitism (Grim *et al.* 2011). Variation in natural or artificial olfactory cues also does not induce egg rejection in American robins (Hauber 2020).

Capture

To assess circulating corticosterone (n=43) and pituitary gene expression for a subset of subjects (n=13), we captured experimentally parasitized females 2 hours after the addition of a model egg. The 2 h time point was chosen because plasma corticosterone in birds typically increases rapidly in the first 30 min to an hour following an exposure to an acute stressor (such as handling or nest disturbance), after which plasma corticosterone titer remains near its maximum if the stressor persists (Abolins-Abols *et al.* 2016; Romero *et al.* 1998; Meddle *et al.* 2003; Breuner *et al.* 2006). For logistical reasons we were not able to remain near the nest to determine the return time of each female, but in this population, females typically arrive back to their nests on average 12 min after being flushed (pers. obs. n=31, standard deviation (SD) = 7.37, range = 2 to 28). Most of the females (86%: 38 out of 43) were flushed from their nests prior the insertion of the model egg (the proportion of flushed females did not differ between the treatments (Fisher's exact test, p=1.0)). The 2-hour time window therefore provided most of the females with an opportunity for a prolonged interaction with the model egg before capture, allowing any corticosterone increase in response to the model eggs to reach its maximum.

Previous research also showed that most female robins' decisions to reject or accept a model egg are not immediate but occur on the timescale of hours rather than minutes (Hauber *et al.* 2019; Scharf *et al.* 2019). Importantly, the only other study we are aware of in *Turdus* thrushes investigating the effect of experimental parasitism on plasma corticosterone detected elevated corticosterone 24+ hours after the addition of the model egg (Ruiz-Raya*et al.* 2018). However, sampling robins at 24 hours following the experimental parasitism without tethering the model eggs would have meant that nearly all the females would have rejected the non-mimetic parasitic eggs (e.g. Hauber et al., 2020b) and that they would therefore have been unstimulated for several hours before capture. The 2-hour time window thus allowed us to catch females during or closely following stimulation by model eggs, and, according to our estimate, allowed sufficient time for any changes in corticosterone in most females reach or remain near peak levels.

We captured females with a 6 m mist net, arranged in a V-shape around the nest tree. The poles and a rolled-up closed mist net were set up immediately before the addition of the model eggs. Two hours after the initial net setup and the addition of the model egg, we unfurled the mist net and captured the female either by flushing it into the net or passively capturing it as it attempted to land on the nest. Setting up the net prior to experimental parasitism enabled rapid set up and capture following the 2-hour mark (n = 42, mean capture time = 15.33 minutes, min = 0, max = 36, SD = 9.19) and allowed us to minimize any changes to corticosterone levels due to nest disturbance. Importantly, we found no association between baseline plasma corticosterone concentration (see below for corticosterone assay methods) and the time it took to catch the female (Pearson's r =-0.16, df=40, p=0.30) or whether the female was flushed from the nest (two-tailed t-test, t=0.15, df=4.64, p=0.88). Therefore, we did not account for these metrics in the statistical models testing the effect of egg type on corticosterone levels (see section below). At the time of capture, we also noted whether the model egg was rejected or still present ("accepted").

Corticosterone sampling and measurement

We sampled 75 µl blood from each subject's brachial vein within 3 min of capture (mean = 157.9 sec, min = 85, max = 216, SD = 30.877; for two birds their blood collection ended after the 3 min mark). The time to end blood collection was not correlated with natural log-transformed baseline corticosterone levels (Pearson's r = 0.03, df = 41, p = 0.87) and we did not account for it in the statistical models.

Blood was kept on ice until centrifugation 2-8 hrs later at +4 °C for 10 min at 8000 rpm. Following centrifugation, blood plasma was removed and stored at -80 °C until analysis. Plasma corticosterone was measured using enzyme immunoassay (Cayman Chemical, catalogue number 501320; Ann Arbor, MI, USA) which has been validated and optimized for American robins (Abolins-Abols & Hauber 2020b). We extracted the non-polar components of plasma using diethyl ether extraction, described in (Abolins-Abols & Hauber 2020b). Briefly, 10 µl plasma was suspended in 200 µl ultrapure water and mixed with 1 ml diethyl ether. After passive phase separation, the mixture was flash-frozen, and the ether phase was decanted. Ether was then evaporated using nitrogen gas at 40 °C. Ether was added to the aqueous portion and decanted two more times. The extract was suspended in 600 µl assay buffer overnight at 4 °C. The concentration of corticoste-

rone in the extract was measured according to the manufacturer's instructions. Each extracted sample was analyzed in triplicate. We measured absorbance at 405 nm using Biotek 800TS plate reader (Winooski, VT, USA) and analyzed the data using an 8-point logistic curve using the Cayman Chemical analysis spreadsheet. The average intraplate coefficient of variation (CV) was 5.78%, while inter-plate CV was 6.19%; we therefore averaged values from the three replicates for each sample and standardized concentrations across places using the mean concentration of a standard control sample, for use in the statistical models.

Gene expression analysis

Immediately following blood collection, a subset (n=13) of females was euthanized using isoflurane overdose, followed by rapid decapitation. The sample size for gene expression analyses was small to limit the number of euthanized wild songbirds. The brain was dissected and reserved for another study. The skull with the embedded pituitary gland was flash-frozen on pulverized dry ice and transferred to a -80 °C freezer within 6 hours. Pituitaries were then rapidly dissected from frozen skulls and returned to -80 °C storage.

For RNA-extraction, the tissue was suspended in 500ul Tri-Reagent (Molecular Research Center, Cincinnati, OH, USA) and homogenized. RNA was then extracted using the manufacturer's protocols, treated with DNase I (New England Biolabs, Ipswich, MA, USA) and purified using QIAGEN RNeasy (Valencia, CA, USA) mini kit.

RNAseq libraries were constructed at the DNA Services laboratory of the Roy J. Carver Biotechnology Center at the University of Illinois at Urbana-Champaign using the TruSeq Stranded RNA Sample Preparation Kit (Illumina San Diego, CA). Briefly, the total RNA was quantitated by Qubit (Life Technologies, Grand Island, NY), then PolyA+ RNA was selected from 1µg of total RNA per sample. PolyA+ RNA was fragmented for 4 minutes at 94C, then first-strand cDNA was synthesized with a random hexamer and SuperScript II (Life Technologies). Double stranded DNA was blunt-ended, 3'-end A-tailed and ligated to unique dual-indexed adaptors. The adaptor-ligated double-stranded cDNA was amplified by PCR for 10 cycles with the Kapa HiFi polymerase (Kapa Biosystems, Woburn, MA). The final libraries were quantitated on Qubit and the average size determined on the AATI Fragment Analyzer (Advanced Analytics, Ames, IA) and diluted to 5nM final concentration. The 5nM dilution was further quantitated by qPCR on a BioRad CFX Connect Real-Time System (Bio-Rad Laboratories, Inc. CA).

The final stranded RNASeq library pool consisting of 13 libraries was sequenced on 1 lane of an Illumina NovaSeq 6000 SP flowcell as paired-reads with 150nt length. The run generated .bcl files which were converted into adaptor-trimmed demultiplexed fastq files using bcl2fastq v2.20 Conversion Software (Illumina, CA).

Heart rate analysis

In 2018 we exposed an additional set of robin females (n=14) to the same binary model egg treatments and measured their heart rate during incubation immediately following the experimental parasitism. We measured heart rate by adopting an approach from Arnold *et al.*(2011) where a microphone-fitted model egg is used to record audio signatures of heartbeats in incubating females through contact with the featherless brood patch. We 3D-printed a custom model eggshell resembling American robin eggs in size and shape using MakerBot Replicator Mini+ 3D printer (MakerBot, Brooklyn, NY, USA). The model shell consisted of two halves, which connected along the long axis of symmetry. The two halves each had an opening on the egg equator – one half had an opening on for a 6 mm microphone, the other had an opening for a headphone cord (Fig. S1a). After printing, each model egg's surface was smoothed using sandpaper. Each egg was then fitted with a unidirectional microphone (PUM-3546L-R, PUI Audio Inc, Dayton, OH, USA). The microphone was soldered to a headphone cable and inserted into the model egg ("top" side of the egg), with the headphone cable leaving the model egg on the opposite ("bottom") side of the egg (Fig. S1a). A white rubber balloon (Walmart, Bentonville, AR, USA) was then stretched over the egg and tied with a fishing line around the headphone cable. The egg was then painted in mimetic robin-blue following the methods outlined above.

To insert the model egg in the nest, we first removed all the robin eggs to prevent damage to them. We then

poked a small hole in the mud-lined bottom of the robin's nest cup through which we passed the headphone cable, leaving the model egg resting on the bottom of the nest with the microphone side facing up (Fig. S1b). The robin eggs were then returned to the nest, typically within 1 min of their removal. One robin egg was removed to avoid changes to the focal subjects' apparent own (robin-sized egg) clutch size. The headphone cord was then connected to a digital sound recorder (Olympus Digital Voice Recorded WS-852, Olympus, Tokyo, Japan).

We first placed the microphone-egg into the focal nests a day before the experimental model egg addition to minimize the disturbance at the nest. We recorded 2 hours of heartbeat audio signatures to check that the model egg was contacting the brood patch and recording audio signatures of heart beats (Fig. S2). One day following the insertion of the microphone egg, we returned to the nest and inserted either mimetic blue or non-mimetic beige cowbird-sized solid nylon model eggs (as described above) in the focal nest and started to record the heart rate again. We returned to the nest 2 hours following the experimental parasitism, removed the experimental model egg and replaced it with the opposite treatment. For example, in nests where a female was experimentally parasitized with a non-mimetic beige egg, the beige egg was removed and a mimetic blue egg was added in its place (and vice versa). Each female was therefore sequentially exposed to both mimetic and non-mimetic treatments, but the order of the treatments was randomized among subjects. Two hours after the addition of the second experimental egg we removed both the experimental and the microphone egg.

We used loud rustling sounds to determine the arrival of the female at the nest cup. We then filtered the heart rate audio recordings to remove sounds above 1 KHz frequencies using Adobe Audition (Adobe, San Jose, CA, USA). We then manually scored the heartbeats using R programming environment using seewave (Sueur *et al.* 2008) and tuneR (Ligges*et al.* 2018) packages following published protocols (Sueur 2018). Heartbeats were then transformed into instantaneous heart rate (beats/sec) using the following formula: 1/i, where i is the interval (sec) between two successive heart beats. Unlike endocrine responses, heart rate is labile and can reflect instantaneous responses to stimuli (Wascher 2021) therefore we limited our analyses to the first 10 minutes following the arrival of the focal female.

Statistical analyses

All analyses were conducted using R programming environment (R Core Team 2017). The effect of egg type (mimetic *vs.* non=mimetic) on corticosterone levels was analyzed using a linear model. Corticosterone levels were natural log-transformed to conform to the expectations of normal distribution (log-transformed corticosterone: Shapiro-Wilk W=0.966, p = 0.238). In addition to the egg type, we included the date of the manipulation as a fixed factor in the analyses, because maternal hormone levels often show strong seasonality (Tyrrell & Cree 1998; Jawor *et al.* 2006; Hauber *et al.* 2020a) and in this study corticosterone showed a significant decrease over the breeding season (see below). We then used a linear model with date as a covariate to test if corticosterone levels differed between females who rejected or accepted a non-mimetic egg at the two-hour mark.

For RNAseq analysis, all paired-end reads were mapped to the Swainson's thrush genome (Accession GCF_-009819885.1) as it was the closest available full genome assembly at the time of this analysis. Paired-end mapping was completed using rsem (Li & Dewey 2011) and bowtie 1.0.0 (Langmead *et al.* 2009) with default parameters. To exclude unexpressed genes and genes not present in robins, only genes with at least 10 identified read counts in over one-third of samples were included for analysis. Differential expression was analyzed using DESeq (Anders & Huber 2010) for differences between treatments and differences between individuals who rejected and those that did not reject the mimic egg. For all analyses, the threshold for significance was set at a false-discovery-rate of 5%.

We calculated instantaneous heart rate each minute, for the first 10 minutes, after the bird arrived at the nest (first audible detection). The average instantaneous heart rates over this 10-minute period were then log-transformed to satisfy assumptions of normality (log-minute averages: Shapiro-Wilk W = 0.992 p-value = 0.058). We then used linear mixed models using R package nlme (Pinheiro *et al.* 2015) to analyze the

effect of model egg color on average heartrate, adding time since arrival, treatment date, and treatment order as covariates, and bird ID as a random factor. Time since arrival was included because robin heart rate was typically high immediately after the arrival at the nest (presumably due to the metabolic demands of flight) following which the heart rate rapidly decreased. To test if individuals differed in the rate of which their heart rate declined following the arrival we asked if addition or random slope terms to the model resulted in a better model fit. However, random slope models had a higher AIC, therefore the final models did not include the random slope term. Only one female rejected the non-mimetic beige egg within the 10 min heart rate recording window and we, therefore, did not include the rejection as a covariate in the model. However, we ran a separate model asking if females that rejected the non-mimetic beige egg within two hours had a higher heart rate in response to the beige eggs compared to females that did not reject these eggs within this time period.

Results

Heart rate showed a significant decrease with time after arrival (LMM, estimate = -0.020, df = 208, t = -10.530, $p < 0.001$), but variation in heart rate was not explained by the treatment date (LMM, estimate = 0.002, df = 11, t = 0.485, p=0.637) or treatment order (LMM, estimate = 0.051, df = 11, t = 0.682, p =0.509). Model egg color type had no statistical effect on incubating females' (n=14) heart rate immediately after return to the nest (LMM, estimate = 0.009, df = 208, t = 0.824, p = 0.411, Fig. 2). Furthermore, initial heart rate did not differ between rejecter and acceptor females during the non-mimetic egg trials (LMM, estimate = -0.053, df = 9, t = -0.875, p = 0.404). Eight out of 14 females rejected the non-mimetic egg within 2 hrs.

Subjects exposed to mimetic or non-mimetic model eggs did not show significant differences in circulating corticosterone concentrations 2 hours after experimental parasitism (LM, estimate: -0.118, t = -0.913, df = 40, p = 0.367; Fig. 2). Plasma corticosterone concentrations decreased across the season (LM, estimate = -0.012, t = -4.301, df = 40, $p < 0.001$). None of the mimetic blue eggs were rejected (n=22) whereas 7 out of 21 (33%) of robins rejected the non-mimetic egg within 2 hours of its addition (Fisher's exact test, p=0.0036). However, plasma corticosterone did not differ between females who rejected or accepted the non-mimetic egg (LM, estimate: -0.008, df = 18, t = -0.044, p = 0.966; Fig. 3).

Prior to false discovery rate (FDR) correction, 312 pituitary-expressed genes were statistically significantly differentially expressed between birds exposed to the mimetic and non-mimetic egg treatments (Supplementary Table 1) two hours following the experimental parasitism. Among these, POMC showed higher expression in pituitary in birds exposed to non-mimetic model eggs (n=6) compared to birds exposed to mimetic eggs (n=7, fold-change = 0.719, p = 0.042, Fig. 4). One of the most significantly differentially expressed genes prior to FDR correction was ATF3, a transcription factor specifically associated with the stress response (Hai *et al.* 1999) , which was upregulated in the birds exposed to non-mimetic eggs (fold change=0.323, $p<0.001$). No genes were significantly differentially expressed between the model egg treatments following FDR correction. POMC expression levels and corticosterone concentrations were not correlated with each other (Spearman's rho = 0.055, df = 11, p = 0.863, Fig. 5).

Discussion

We found that experimental nest parasitism does not elevate circulating plasma corticosterone in a typically egg-rejecting host of an obligate avian brood parasite 2 hours following the addition of the model eggs. However, at the same time point, the expression of the HPA axis-relevant POMC gene and the stress-related transcription factor ATF3 in the pituitary was elevated in birds exposed to the non-mimetic model egg compared to the individuals exposed to the mimetic egg, although these differences were not significant after false discovery rate correction. FDR limits the type-I error in datasets with a large number of comparisons. However, given that we were mainly interested in a few HPA-axis candidate genes in the pituitary, we also report the uncorrected p-value, while still acknowledging the possibility of this result being a false positive. We detected no changes in the immediate orienting heart rate responses of incubating robins between our model egg-color treatments.

These results are consistent with a delayed activation of the HPA axis in response to brood parasitism. In this study, we measured POMC mRNA levels in the pituitary, not ACTH itself. Although POMC gene expression can be upregulated within 30 minutes of CRH stimulation (Levin *et al.* 1989), translation and posttranslational modification of the POMC peptide into ACTH likely take time, as POMC needs to be packaged in vesicles where it is cleaved into daughter peptides (Pritchard & White 2007). Thus, it is possible that a POMC-related corticosterone increase occurs after our 2-hour mark. Indeed, we found no association between POMC expression and corticosterone levels at the time of sampling our subjects. Alternatively, it is possible that at 2 hrs we already missed a rapid elevation of circulating glucocorticoids following exposure to the non-mimetic model egg and the p-value based detection of the increased POMC gene expression data is simply a statistical artifact. ATF3, which was upregulated in birds exposed to non-mimetic eggs, is typically upregulated in cells experiencing physiological stress (Hai*et al.* 1999), although ATF3 expression can also be induced by psychological restraint stressor (Green *et al.* 2008). The upregulation of ATF3 in the pituitary in response to a brood-parasitic egg thus supports the hypothesis that parasitic eggs are perceived as stressful by the hosts, although the role of ATF3 expression in the pituitary is unclear.

In a different study investigating changes in corticosterone in response to parasitic eggs in a congeneric species (European blackbird) an increase in baseline corticosterone was detected 24+ hrs following experimental parasitism with a non-mimetic egg relative to control treatment (Ruiz-Raya *et al.* 2018). This suggests that distinctive parasitic eggs may cause a slow but continuous increase in HPA activity over multiple hours. Consistent with this interpretation, we found no difference in the heart rate between the non-mimetic and mimetic egg treatments immediately following the arrival of the female at the nest. Heart rate can serve as a rapid, real-time indicator of acute stress response (Cyr *et al.* 2009), and the lack of differences in the heart rate immediately after encountering the egg suggests that parasitic stimuli do not cause an acute stress response.

A low, but long-term increase in HPA activity may have functional significance in the context of modulating egg rejection, even if it is not statistically at a single time point of sampling. For example, (Abolins-Abols & Hauber 2020a) found that long-term (overnight) suppression of glucocorticoid synthesis increases the acceptance rate of parasitic eggs by American robins. If natural glucocorticoid release in response to parasitic eggs stimulates egg rejection, then minimal but long-term upregulation of the HPA axis in response to parasitic egg stimuli is consistent with the timeframe of rejection of parasitic eggs by American robins. For example, >90% of robins reject non-mimetic beige cowbird-sized eggs within 5 days (Luro *et al.*2018), 2 days (Hauber *et al.* 2019), and even 1 day (Hauber*et al.* 2020b), but only 33% (7 out of 21) birds rejected the same non-mimetic egg type within 2 hours in this study (also see Scharf et al., 2019). However, individual variation in glucocorticoid levels in this study did not predict egg rejection: circulating corticosterone at our single time-point of sampling did not differ between females who accepted or rejected the non-mimetic beige eggs (Fig. 3).

The few steroid-focused endocrine studies on physiological responses to parasitic egg stimuli so far thus paint a complicated picture. On one hand, experimental studies now show that glucocorticoids (Abolins-Abols & Hauber 2020a) can mediate egg rejection. On the other hand, the HPA axis may only show weak gradual activation in response to exposure to brood parasitic eggs and may have no discernible effect on egg rejection. It is possible that egg rejection by brood parasite hosts may be affected only by pronounced changes in the HPA activity (such as those due to experimental manipulation of hormone levels or intense stressors) and not by weak HPA activation in response to brood parasitism. Furthermore, the relationship between stress physiology and egg rejection may be species-specific. For example, in incubating prothonotary warblers (*Protonotaria citrea*), a non-rejecter host species, experimental parasitism with either non-mimetic or mimetic eggs had no effect on glucocorticoid levels relative to non-parasitized controls (Scharf *et al.* 2021). On the other hand, in yellow warblers (*Setophaga petechia*), a species that frequently but variably abandons its nest if parasitized by brown-headed cowbirds, individuals with higher circulating corticosterone levels were more likely to desert an experimentally parasitized nest relative to non-parasitized controls (Turcotte-van de Rydt *et al.* 2022).

Methodologically, especially when studying freely behaving wild animals, we are still limited by our inability to measure the minute-by minute dynamics in glucocorticoid synthesis and release, as well as our inability to accurately assess long-term subtle changes in glucocorticoids (and other hormones) that could cause a behavioral change. To integrate our findings into a unified framework, future experiments should characterize the full-time course and magnitude of HPA axis activation in response to brood parasitism across acceptor and rejector species. Additionally, dose-response studies, such as those inducing small and short-term changes in hormone levels (e.g., Vitousek et al. 2018) are necessary to test the sensitivity of host behavior to hormones. Finally, future studies must expand beyond the corticosterone paradigm and test the effect of other hormones, such as prolactin, on host behaviors (Abolins-Abols & Hauber 2018; Ruiz-Raya *et al.*2021), as well as stress-response at the cellular level (Ruiz-Raya*et al.* 2022). Together, these studies will allow us to more fully understand the significance of stress signaling in the ecology and evolution of host defenses.

Conclusions

We tested one of the predictions of the "stress-mediated egg rejection" hypothesis, which predicts that hosts should mount physiological stress response when they perceive cues of brood parasitism. Our results are partially consistent with this prediction: while we show that POMC expression is elevated in birds exposed to non-mimetic eggs compared to mimetic eggs two hours after experimental parasitism, plasma corticosterone levels did not differ between the treatments at this timepoint, and experimental parasitism with non-mimetic eggs did not affect heart rate. These findings suggest that to understand the applicability and the ecological relevance of stress-mediated egg rejection, we need to address the diversity and subtlety of the stress-response and its effects on behavior in egg rejecter hosts of brood parasites.

Data availability Statement: Data will be made publicly available following the acceptance of this manuscript.

Competing Interests Statement: All authors have no conflicts of interest to declare.

Author Contributions: MA-A, MEH, and DH conceived the study, MA-A and BS conducted the field and laboratory work, GB provided expertise for laboratory work, and MA-A, MP, and MH analyzed data. MA-A wrote the manuscript with extensive input from all co-authors.

Acknowledgements: This research was permitted by institutional (University of Illinois IACUC # 17049), state (Illinois Department of Natural Resources # NH18.6220), and federal (US Fish and Wildlife Service #MB08861A-2) agencies. Funding was provided by the Harley Jones Van Cleave Professorship to MEH, the Katma Award of the American Ornithological Society (to MA-A and MEH), and the LIU-Post summer internship program (to BS and DH). Additional support was provided by the Wissenschaftskolleg zu Berlin, Germany and the Center for Advanced Study at the University of Illinois (to MEH).

Literature cited

Abolins-Abols, M. & Hauber, M.E. (2018) Host defences against avian brood parasitism: an endocrine perspective. *Proc R Soc B Biol Sci***285** , 20180980.

Abolins-Abols, M. & Hauber, M.E. (2020a) Endocrine regulation of egg rejection in an avian brood parasite host. *Biol Lett* **16** , 20200225.

Abolins-Abols, M. & Hauber, M.E. (2020b) Proximate predictors of variation in egg rejection behavior by hosts of avian brood parasites.*J Comp Psychol* **134** , 412–422.

Abolins-Abols, M., Hope, S.F. & Ketterson, E.D. (2016) Effect of acute stressor on reproductive behavior differs between urban and rural birds.*Ecol Evol* **6** , 6546–6555.

Anders, S. & Huber, W. (2010) Differential expression analysis for sequence count data. *Genome Biol* **11** , R106.

Angelier, F. & Chastel, O. (2009) Stress, prolactin and parental investment in birds: A review. *Gen Comp Endocrinol* **163** , 142–148.

Angelier, F., Clément-Chastel, C., Welcker, J., Gabrielsen, G.W. & Chastel, O. (2009) How does corticosterone affect parental behaviour and reproductive success? A study of prolactin in black-legged kittiwakes.*Funct Ecol* **23** , 784–793.

Arnold, J.M., Ordonez, R., Copeland, D.A., Nathan, R., Scornavacchi, J.M., Tyerman, D.J. & Oswald, S.A. (2011) Simple and inexpensive devices to measure heart rates of incubating birds. *J Field Ornithol* **82** , 288–296.

Brain, P.F. & Evans, A.E. (1977) Acute influences of some ACTH-related peptides on fighting and adrenocortical activity in male laboratory mice. *Pharmacol Biochem Behav* **7** , 425–433.

Breuner, C.W., Lynn, S.E., Julian, G.E., Cornelius, J.M., Heidinger, B.J., Love, O.P., Sprague, R.S., Wada, H. & Whitman, B. a. (2006) Plasma-binding globulins and acute stress response. *Horm Metab Res* **38** , 260–8.

Briskie, J.V., Sealy, S.G. & Hobson, K.A. (1992) Behavioral defenses against avian brood parasitism in sympatric and allopatric host populations. *Evolution* **46** , 334–340.

Canniff, L., Dainson, M., Lopez, A.V., Hauber, M.E., Grim, T., Samaš, P. & Hanley, D. (2018) Probing the limits of egg recognition using egg rejection experiments along phenotypic gradients. *JoVE J Vis Exp*e57512–e57512.

Cyr, N.E., Dickens, M.J. & Romero, L.M. (2009) Heart rate and heart-rate variability responses to acute and chronic stress in a wild-caught passerine bird. *Physiol Biochem Zool* **82** , 332–344.

Davies, N.B. & Brooke, M. de L. (1988) Cuckoos versus reed warblers: Adaptations and counteradaptations. *Anim Behav* **36** , 262–284.

Davies, N.B. & Brooke, M.D.L. (1989) An experimental study of co-evolution between the cuckoo, cuculus canorus, and its hosts. I. Host Egg Discrimination. *J Anim Ecol* **58** , 207–224.

Deng, Q., Riquelme, D., Trinh, L., Low, M.J., Tomić, M., Stojilkovic, S. & Aguilera, G. (2015) Rapid glucocorticoid feedback inhibition of acth secretion involves ligand-dependent membrane association of glucocorticoid receptors. *Endocrinology* **156** , 3215–3227.

Feeney, W.E., Welbergen, J.A. & Langmore, N.E. (2014) Advances in the study of coevolution between avian brood parasites and their hosts.*Annu Rev Ecol Evol Syst* **45** , 227–246.

Gallo-Payet, N. (2016) 60 YEARS OF POMC: Adrenal and extra-adrenal functions of ACTH. *J Mol Endocrinol* **56** , T135–T156.

Green, T.A., Alibhai, I.N., Unterberg, S., Neve, R.L., Ghose, S., Tamminga, C.A. & Nestler, E.J. (2008) Induction of activating transcription factors (ATFs) ATF2, ATF3, and ATF4 in the nucleus accumbens and their regulation of emotional behavior. *J Neurosci***28** , 2025–2032.

Grim, T., Samaš, P. & Hauber, M.E. (2014) The repeatability of avian egg ejection behaviors across different temporal scales, breeding stages, female ages and experiences. *Behav Ecol Sociobiol***68** , 749–759.

Grim, T., Samaš, P., Moskát, C., Kleven, O., Honza, M., Moksnes, A., Røskaft, E. & Stokke, B.G. (2011) Constraints on host choice: why do parasitic birds rarely exploit some common potential hosts? *J Anim Ecol* **80** , 508–518.

Hahn, D.C., Wingfield, J.C., Fox, D.M., Walker, B.G. & Thomley, J.E. (2017) Maternal androgens in avian brood parasites and their hosts: Responses to parasitism and competition? *Gen Comp Endocrinol***240** , 143–152.

Hai, T., Wolfgang, C.D., Marsee, D.K., Allen, A.E. & Sivaprasad, U. (1999) ATF3 and stress responses. *Gene Expr* **7** , 321–335.

Harno, E., Gali Ramamoorthy, T., Coll, A.P. & White, A. (2018) POMC: The physiological power of hormone processing. *Physiol Rev***98** , 2381–2430.

Hauber, M.E. (2003a) Egg-capping is a cost paid by hosts of interspecific brood parasites. *The Auk* **120** , 860–865.

Hauber, M.E. (2003b) Hatching asynchrony, nestling competition, and the cost of interspecific brood parasitism. *Behav Ecol* **14** , 227–235.

Hauber, M.E. (2020) Natural and artificial scents do not increase egg rejection rates of model brood parasitic eggs by American robins (*Turdus migratorius*). *Acta Zool Acad Sci Hung* **66** , 309–317.

Hauber, M.E., Abolins-Abols, M., Kim, C.R. & Paitz, R.T. (2020a) Inter-individual variation in anti-parasitic egg rejection behavior: a test of the maternal investment hypothesis. *Integr Org Biol***2** .

Hauber, M.E., Dainson, M., Luro, A., Louder, A.A. & Hanley, D. (2019) When are egg-rejection cues perceived? A test using thermochromic eggs in an avian brood parasite host. *Anim Cogn* **22** , 1141–1148.

Hauber, M.E., Hoover, J.P., Rhodes, O.R., Ducay, R.L. & Hanley, D. (2020b) The blunt pole is not a source of more salient recognition cues than the sharp pole for the rejection of model eggs by American robins (*Turdus migratorius*). *J Vertebr Biol* **70** , 20111.1.

Hauber, M.E., Kim, C.R., Goethe, C. & Hanley, D. (2020c) Self-referent phenotype matching is a poor predictor of egg rejection by American robins. *J Field Ornithol* **91** , 254–262.

Hauber, M.E., Moskát, C. & Bán, M. (2006) Experimental shift in hosts' acceptance threshold of inaccurate-mimic brood parasite eggs. *Biol Lett* **2** , 177–80.

Hauber, M.E., Pearson, H.E., Reh, A. & Merges, A. (2002) Discrimination between host songs by brood parasitic brown-headed cowbirds (*Molothrus ater*). *Anim Cogn* **5** , 129–137.

Honza, M., Procházka, P., Morongová, K., Čapek, M. & Jelínek, V. (2011) Do nest light conditions affect rejection of parasitic eggs? a test of the light environment hypothesis: ambient nest light and egg rejection.*Ethology* **117** , 539–546.

Igic, B., Nunez, V., Voss, H.U., Croston, R., Aidala, Z., López, A.V., Van Tatenhove, A., Holford, M.E., Shawkey, M.D. & Hauber, M.E. (2015) Using 3D printed eggs to examine the egg-rejection behaviour of wild birds. *PeerJ* **3** , e965.

Jawor, J.M., McGlothlin, J.W., Casto, J.M., Greives, T.J., Snajdr, E.A., Bentley, G.E. & Ketterson, E.D. (2006) Seasonal and individual variation in response to GnRH challenge in male dark-eyed juncos (*Junco hyemalis*). *Gen Comp Endocrinol* **149** , 182–189.

Ketterson, E.D., Atwell, J.W. & McGlothlin, J.W. (2009) Phenotypic integration and independence: Hormones, performance, and response to environmental change. *Integr Comp Biol* **49** , 365–79.

Ketterson, E.D. & Nolan, V.Jr. (1999) Adaptation, exaptation, and constraint: a hormonal perspective. *Am Nat* **154** , S4–S25.

Krüger, O. (2007) Cuckoos, cowbirds and hosts: adaptations, trade-offs and constraints. *Philos Trans R Soc Lond B Biol Sci* **362** , 1873–86.

Langmead, B., Trapnell, C., Pop, M. & Salzberg, S.L. (2009) Ultrafast and memory-efficient alignment of short DNA sequences to the human genome. *Genome Biol* **10** , R25.

Levin, N., Blum, M. & Roberts, J.L. (1989) Modulation of basal and corticotropin-releasing factor- stimulated proopiomelanocortin gene expression by vasopressin in rat anterior pituitary.*Endocrinology* **125** , 2957–2966.

Li, B. & Dewey, C.N. (2011) RSEM: accurate transcript quantification from RNA-Seq data with or without a reference genome. *BMC Bioinformatics* **12** , 323.

Ligges, U., Krey, S., Mersmann, O. & Schnackenberg, S. (2018)*tuneR: Analysis of Music and Speech* .

Lotem, A., Nakamura, H. & Zahavi, A. (1995) Constraints on egg discrimination and cuckoo-host co-evolution. *Anim Behav***49** , 1185–1209.

Luro, A.B. & Hauber, M.E. (2017) A test of the nest sanitation hypothesis for the evolution of foreign egg rejection in an avian brood parasite rejecter host species. *Sci Nat* **104** , 14.

Luro, A.B., Igic, B., Croston, R., López, A.V., Shawkey, M.D. & Hauber, M.E. (2018) Which egg features predict egg rejection responses in American robins? Replicating Rothstein's (1982) study. *Ecol Evol***8** , 1673–1679.

MacDougall-Shackleton, S.A., Bonier, F., Romero, L.M. & Moore, I.T. (2019) Glucocorticoids and "stress" are not synonymous. *Integr Org Biol* **1** , obz017.

Mark, M.M. & Rubenstein, D.R. (2013) Physiological costs and carry-over effects of avian interspecific brood parasitism influence reproductive tradeoffs. *Horm Behav* **63** , 717–722.

Meddle, S.L., Owen-Ashley, N.T., Richardson, M.I. & Wingfield, J.C. (2003) Modulation of the hypothalamic-pituitary-adrenal axis of an Arctic-breeding polygynandrous songbird, the Smith's longspur,*Calcarius pictus* . *Proc Biol Sci* **270** , 1849–1856.

Medina, I. & Langmore, N.E. (2016) The evolution of acceptance and tolerance in hosts of avian brood parasites. *Biol Rev***91** , 569–577.

Miller, R.E. & Ogawa, N. (1962) The effect of adrenocorticotrophic hormone (ACTH) on avoidance conditioning in the adrenalectomized rat.*J Comp Physiol Psychol* **55** , 211–213.

Moksnes, A. & Røskaft, E. (1989) Adaptations of meadow pipits to parasitism by the common cuckoo. *Behav Ecol Sociobiol***24** , 25–30.

Moksnes, A., Røskaft, E. & Braa, A.T. (1991) Rejection behavior by common cuckoo hosts towards artificial brood parasite eggs. *The Auk* **108** , 348–354.

Pinheiro, J., Bates, D., DebRoy, S., Sarkar, D., & R Core Team. (2015) nlme: Linear and Nonlinear Mixed Effects Models.

Pritchard, L.E. & White, A. (2007) Neuropeptide processing and its impact on melanocortin pathways. *Endocrinology* **148** , 4201–4207.

R Core Team. (2017) *R: a language and environment for statistical computing* . R Foundation for Statistical Computing, Vienna, Austria.

Romero, L.M. (2006) Seasonal changes in hypothalamic-pituitary-adrenal axis sensitivity in free-living house sparrows (*Passer domesticus*). *Gen Comp Endocrinol* **149** , 66–71.

Romero, L.M. & Butler, L.K. (2007) Endocrinology of Stress. *Int J Comp Psychol* **20** , 89–95.

Romero, L.M. & Gormally, B.M.G. (2019) How truly conserved is the "well-conserved" vertebrate stress response? *Integr Comp Biol***59** , 273–281.

Romero, L.M., Soma, K.K. & Wingfield, J.C. (1998) Hypothalamic-pituitary-adrenal axis changes allow seasonal modulation of corticosterone in a bird. *Am J Physiol - Regul Integr Comp Physiol* **274** , R1338–R1344.

Rosvall, K.A. (2013) Proximate perspectives on the evolution of female aggression: good for the gander, good for the goose? *Philos Trans R Soc B Biol Sci* **368** , 20130083.

Rothstein, S.I. (1975) Mechanisms of avian egg-recognition: Do birds know their own eggs? *Anim Behav* **23** , 268–278.

Rothstein, S.I. (1982) Mechanisms of avian egg recognition : which egg parameters elicit responses by rejecter species? *Behav Ecol Sociobiol* **11** , 229–239.

Ruiz-Raya, F. (2021) Ecophysiology of egg rejection in hosts of avian brood parasites: new insights and perspectives. *Curr Zool*zoab042.

Ruiz-Raya, F., Abaurrea, T., Vigo, R. & Soler, M. (2022) Physiological stress responses to non-mimetic model brood parasite eggs: leukocyte profiles and heat-shock protein Hsp70 level.

Ruiz-Raya, F., Ibanez-Alamo, J.D., Parenteau, C., Chastel, O. & Soler, M. (2021) Prolactin mediates behavioural rejection responses to avian brood parasitism. *J Exp Biol* **224** .

Ruiz-Raya, F. & Soler, M. (2017) Phenotypic Plasticity in Egg Rejection: Evidence and Evolutionary Consequences. In Soler, M. (ed),*Avian Brood Parasitism: Behaviour, Ecology, Evolution and Coevolution* , Fascinating Life Sciences, Springer International Publishing, Cham, pp. 449–471.

Ruiz-Raya, F. & Soler, M. (2020) Signal detection and optimal acceptance thresholds in avian brood parasite–host systems: implications for egg rejection. *Philos Trans R Soc B Biol Sci***375** , 20190477.

Ruiz-Raya, F., Soler, M., Abaurrea, T., Chastel, O., Roncalli, G. & Ibanez-Alamo, J.D. (2018) Hormonal responses to non-mimetic eggs: is brood parasitism a physiological stressor during incubation? *Behav Ecol Sociobiol* **72** , 153.

Rutledge, S., Carr, D.E., Hauber, M.E. & Hanley, D. (2021) Best of a bad job or masters of illusion: Do nest light conditions make the eggs of brood parasitic brown-headed cowbirds (*Molothrus ater*) more similar to the eggs of their hosts? *Ethology* **127** , 117–124.

Scharf, H.M., Abolins-Abols, M., Stenstrom, K.H., Tolman, D.T., Schelsky, W.M. & Hauber, M.E. (2021) Exposure to a mimetic or non-mimetic model avian brood parasite egg does not produce differential glucocorticoid responses in an egg-accepter host species. *Gen Comp Endocrinol* **304** , 113723.

Scharf, H.M., Stenstrom, K., Dainson, M., Benson, T.J., Fernandez-Juricic, E. & Hauber, M.E. (2019) Mimicry-dependent lateralization in the visual inspection of foreign eggs by American robins. *Biol Lett* **15** , 20190351.

Soler, M. (2014) Long-term coevolution between avian brood parasites and their hosts. *Biol Rev* **89** , 688–704.

Soler, M. (ed). (2017) *Avian brood parasitism: Behaviour, ecology, evolution and coevolution* . Springer, Cham, Switzerland.

Soler, M. & Moller, A.P. (1990) Duration of sympatry and coevolution between the great spotted cuckoo and its magpie host. *Nature***343** , 748–750.

Stokke, B.G., Moksnes, A. & Roskaft, E. (2005) The enigma of imperfect adaptations in hosts of avian brood parasites. *Ornithol Sci***4** , 17–29.

Sueur, J. (2018) *Sound analysis and synthesis with R* . Springer.

Sueur, J., Aubin, T. & Simonis, C. (2008) Seewave, a free modular tool for sound analysis and synthesis. *Bioacoustics* **18** , 213–226.

Turcotte-van de Rydt, A.V., Petalas, C., Sblendorio, J.M., Pearl, C.A., Gill, S.A. & Guigueno, M.F. (2022) Clutch abandoning parasitised yellow warblers have increased circulating corticosterone with no effect of past corticosterone or differences in egg maculation characteristics.*Front Ecol Evol* **10** .

Tyrrell, C.L. & Cree, a. (1998) Relationships between corticosterone concentration and season, time of day and confinement in a wild reptile (tuatara, *Sphenodon punctatus*). *Gen Comp Endocrinol***110** , 97–108.

Vitousek, M.N., Taff, C.C., Ardia, D.R., Stedman, J.M., Zimmer, C., Salzman, T.C. & Winkler, D.W. (2018) The lingering impact of stress: brief acute glucocorticoid exposure has sustained, dose-dependent effects on reproduction. *Proc R Soc B Biol Sci* **285** , 20180722.

Wascher, C.A.F. (2021) Heart rate as a measure of emotional arousal in evolutionary biology. *Philos Trans R Soc B Biol Sci* **376** , 20200479.

Williams, T.D. (2008) Individual variation in endocrine systems: moving beyond the "tyranny of the Golden Mean." *Philos Trans R Soc Lond B Biol Sci* **363** , 1687–1698.

Zhang, J., Moller, A.P., Yan, D., Li, J. & Deng, W. (2021) Egg rejection changes with seasonal variation in risk of cuckoo parasitism in Daurian redstarts, *Phoenicurus auroreus* . *Anim Behav***175** , 193–200.

Figure captions

Figure 1

Experimental parasitism treatments: a) mimetic robin-blue model egg in a natural robin nest; b) a non-mimetic beige model egg in a natural robin nest.

Figure 2

The effect of model egg treatment on heart rate. Heart rate following addition of non-mimetic beige eggs did not differ from the heart rate following addition of mimetic blue egg in a separate set of experimentally parasitized females (n=21). Circles show time-specific average heart rate within a treatment, error bars indicate standard error. Black dots show time-specific average heart rate for individual females.

Figure 3

The effect of model egg treatment on corticosterone levels. Females experimentally parasitized with non-mimetic beige eggs (n=21) did not differ in baseline corticosterone levels compared to females experimentally parasitized with mimetic blue eggs (n=22). Black dots indicate females that had had not rejected the egg within two hours, while red dots indicate females that rejected the egg. Within the non-mimetic egg treatment, females that rejected and accepted the eggs did not differ in their corticosterone levels.

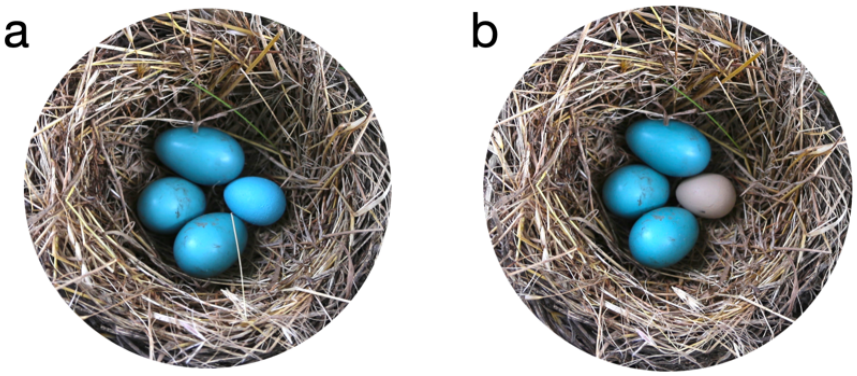
Figure 4

The effect of model egg treatment on POMC expression in the pituitary in a subset of female American robins. Prior to FDR correction, females experimentally parasitized with non-mimetic beige eggs (n=6) showed higher POMC expression compared to females experimentally parasitized with mimetic blue eggs (n=7). Black dots indicate females that had had not rejected the egg within two hours, while red dots indicate females that rejected the egg. POMC expression differences were not significant after FDR correction.

Figure 5

POMC expression does not predict corticosterone levels two hours following experimental parasitism (n=13) of American robins. Shaded area indicates the 95% confidence interval.

Figure 1



Hosted file

image2.wmf available at https://authorea.com/users/441305/articles/570841-patterns-of-stress-response-to-foreign-eggs-by-a-rejecter-host-of-an-obligate-avian-brood-parasite

Figure 2

Figure 3

Hosted file

image3.wmf available at https://authorea.com/users/441305/articles/570841-patterns-of-stress-response-to-foreign-eggs-by-a-rejecter-host-of-an-obligate-avian-brood-parasite

Figure 4

Hosted file

image4.wmf available at https://authorea.com/users/441305/articles/570841-patterns-of-stress-response-to-foreign-eggs-by-a-rejecter-host-of-an-obligate-avian-brood-parasite

Figure 5

Hosted file

image5.wmf available at https://authorea.com/users/441305/articles/570841-patterns-of-stress-response-to-foreign-eggs-by-a-rejecter-host-of-an-obligate-avian-brood-parasite