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Research paper

An experimental test of the effect of brood size on glucocorticoid responses, parental investment, and offspring phenotype

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ABSTRACT

Because elevated glucocorticoid levels can impair reproduction, populations or species that engage in particularly valuable reproductive attempts may down-regulate the glucocorticoid stress response during reproduction (the brood value hypothesis). It is not clear, however, whether individuals rapidly modulate glucocorticoid responses based on shifting cues about the likelihood of reproductive success. By manipulating brood size to create broods that differed in potential value, we tested whether female barn swallows (*Hirundo rustica*) rapidly modulated the glucocorticoid stress response to promote investment in high-value broods, and whether nestling phenotype was influenced by treatment. Within-individual changes in female corticosterone, body mass, and measures of oxidative stress were unrelated to brood size treatment. Standard offspring provisioning rate did not differ across treatments; however, in the presence of a model predator, females raising enlarged broods maintained higher offspring feeding rates relative to control broods. Brood size did influence nestling phenotype. Nestlings from enlarged broods had lower body mass and higher baseline corticosterone than those from reduced broods. Finally, in adult females both baseline and stress-induced corticosterone were individually repeatable. Thus, while under moderately challenging environmental conditions brood size manipulations had context-dependent effects on parental investment, and influenced nestling phenotype, maternal glucocorticoid levels were not modulated based on brood value but were individually consistent features of phenotype during breeding.

1. Introduction

Life history theory predicts that organisms engaged in particularly valuable reproductive attempts will increase reproductive investment, even if doing so may impact future reproductive potential (Drent and Daan, 1980; Stearns, 1992; Trivers, 1972). Experimental manipulations of brood value confirm that parents often, but not always, increase investment in response to perceived changes in brood value (Ardia, 2007; Dijkstra et al., 1990; Nur, 1984; Ridgway, 1989). However, the physiological mediators of this rapid flexibility are not well-understood. Glucocorticoid hormones mediate behaviors central to both reproduction and self-maintenance (Wingfield and Sapolsky, 2003). At baseline levels, glucocorticoids function as metabolic regulators, and mediate behaviors related to energy intake (Landys et al., 2006). When organisms are exposed to stressors glucocorticoid levels rise rapidly, and mediate changes in the expression of behavior and physiology related to the response to and recovery from challenges, including the suppression of reproduction (Wingfield and Sapolsky, 2003). The modulation of glucocorticoid responses during breeding may therefore be an important mechanism enabling flexible reproductive investment (Hau et al., 2016; Lendvai et al., 2007; Taff and Vitousek, 2016; Wingfield et al., 1998; Wingfield and Sapolsky, 2003). Within-population variation in the glucocorticoid levels of individuals facing a standardized stressor – which is often substantial (Cockrem, 2013) – can predict survival (Angelier et al., 2009; Blas et al., 2007) and reproductive success (Patterson et al., 2014; Vitousek et al., 2014). Yet the extent to which relationships between glucocorticoids and fitness result from relatively fixed differences between individuals, or reflect context-dependent within-individual plasticity, is not well-understood (Hau et al., 2016; Taff and Vitousek, 2016).

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Glucocorticoid responses are individually consistent in some contexts (Cockrem et al., 2009; Small and Schoech, 2015; Vitousek and Romero, 2013). Evidence from both artificial selection studies and natural populations suggests that stress-induced glucocorticoid levels are moderately heritable traits (Evans et al., 2006; Jenkins et al., 2014; Pottinger and Carrick, 1999; Satterlee and Johnson, 1988). Consistent individual differences could also result from the sometimes permanent effects of early life experience on endocrine function (Farine et al., 2015; Spencer et al., 2009; Wada, 2014; Zimmer and Spencer, 2014). Yet the response to stress can also exhibit reversible phenotypic flexibility based on ecological (e.g., predator presence: Clinchy et al., 2011), social (e.g., dominance status: Gesquiere et al., 2011), and life history context (e.g., reproductive stage: Vitousek et al., 2010). Identifying the factors that induce phenotypic flexibility in the stress response, and the extent to which this flexibility may itself be individually consistent (Lendvai et al., 2014; Taff and Vitousek, 2016), will advance our understanding of how glucocorticoid levels are regulated, and how circulating glucocorticoids may mediate life-history trade-offs.

Because high glucocorticoid levels can impair reproduction, it may be adaptive to suppress the glucocorticoid stress response when engaged in a particularly valuable reproductive attempt (one that constitutes a greater proportion of future reproductive potential: Trivers, 1972; Heidinger et al., 2006; Wingfield and Sapolsky, 2003). A phylogenetic comparative analysis in birds found some evidence that species with higher value broods mount a weaker glucocorticoid response to a standardized stressor during reproduction (the brood value hypothesis: Bókony et al., 2009). In accordance with the brood value hypothesis, older common terns (*Sterna hirundo*), which have reduced future reproductive potential and thus produce clutches with higher brood value, elevate their corticosterone (the primary avian glucocorticoid) less in response to standardized stressors during breeding (Heidinger et al., 2006, 2010). Likewise, seasonally breeding Eurasian hoopoe (*Upupa epops*) rearing their second and final clutch of the season mount weaker corticosterone stress responses than during earlier nesting attempts (Schmid et al., 2013). But whether individuals rapidly modulate their hormonal stress responses based on shifting cues about potential reproductive gain (Lendvai et al., 2007), is not clear. Previous studies in which offspring number was manipulated to influence reproductive value had differing outcomes: house sparrows (*Passer domesticus*) rearing experimentally enlarged broods reduced their glucocorticoid stress response (Lendvai et al., 2007), but in response to a similar manipulation, smallmouth bass (*Micropterus dolomieu*) did not (O'Connor et al., 2011). Parental investment is typically scaled with brood size in these species (Hegner and Wingfield, 1987; Ridgway, 1989). However, while the response to brood size manipulations can differ across populations and years (Ardia, 2007; Hainstock et al., 2010), neither of these studies directly measured whether parental investment changed in accordance with the brood value hypothesis. Thus, it is not possible to determine whether these contrasting results stem from species differences, or reflect context-dependent differences in the flexibility of both glucocorticoids and parental behavior.

By manipulating brood size in barn swallows (*Hirundo rustica erythrogaster*), we tested whether females flexibly modulate both their response to stress and reproductive investment to promote investment in larger broods, and whether rearing environment (brood size) influences offspring phenotype. If the response to stress is flexibly modulated based on potential reproductive gain, we predicted that females raising enlarged broods would decrease their corticosterone stress response, and increase offspring provisioning rates – both under normal conditions and in the presence of a model predator. We also predicted that this increased reproductive investment would impair the ability to resist oxidative stress by depleting antioxidant capacity, and possibly also elevating plasma oxidative damage (Costantini et al., 2014; Vitousek et al., 2016). We also predicted that females rearing enlarged broods would elevate their baseline glucocorticoid levels, either as an adaptive response to facilitate the increased workload necessary to rear additional offspring, or as a reflection of the increased challenges faced by these females (Bonier et al., 2011). Alternatively, if corticosterone levels are not affected by changes in perceived reproductive potential imposed by brood size manipulations, we predicted that underlying differences between females in circulating corticosterone would result in significant individual repeatability of baseline and stress-induced corticosterone across reproductive sub-stages.

Experimentally manipulating offspring number influences not only parents' perception of brood value, but also the environment in which offspring develop. These effects are likely to be particularly strong if the energetic intake of developing offspring is influenced by nest or litter size, as happens when parents are not fully able to compensate for changes in offspring number. Many previous studies have found that brood size can influence offspring size, physiological state, and secondary sexual traits (Burness et al., 2000; Ilmonen et al., 2003; Kozlowski and Ricklefs, 2011; Musgrove and Wiebe, 2016). While developmental environment can have lifelong effects on the response to challenges (the developmental programming hypothesis: Spencer et al., 2009; Schmidt et al., 2014; Zimmer and Spencer, 2014), few studies have addressed whether stressors associated with brood size manipulations influence the glucocorticoid stress responses of offspring. We therefore also tested the hypothesis that by altering early life environment, brood size manipulations would influence nestling morphology and physiology. We predicted that nestlings from experimentally enlarged broods would be more energetically limited than nestlings in control or reduced broods, and that this chronic stressor would result in nestlings with lower body mass and higher baseline corticosterone levels prior to fledging.

2. Material and methods

2.1. Capture and experimental methods

Experiments were conducted in barn swallows breeding across Boulder, Weld, and Jefferson Counties, Colorado in 2010. Adults were captured either by hand off of nests or by rapidly flushing them into a mist net. During the first capture, adults were banded with numbered and colored bands, and following release, observations at breeding sites were used to trace banded adults to specific nests. Nests were monitored daily, and the first broods of marked females were included in the study if they had a brood size of at least 3 nestlings (range: 3–6; mean ± SE: 4.14 ± 0.11; clutch size range: 4–6, mean ± SE: 4.66 ± 0.07). On the day of hatch, nests ($n = 60$) were grouped according to hatch date and randomly assigned to a treatment group: enlarged ($n = 21$), reduced ($n = 21$), or control ($n = 18$). On the following day (day 1), two nestlings were transferred from reduced nests into enlarged nests. Control nestlings were removed from the nest and handled, but were not transferred. Transferred birds were marked by coloring the tarsus with a non-toxic marker until the nestlings were of sufficient size for banding, which typically occurred on day 5. Nest failures induced slight decreases in sample sizes over the course of the study. Two nests (one control, one reduced) failed before adults were targeted for recapture (which began on day 6 post-hatch), and six additional nests (one enlarged, two control, three reduced) failed before nestlings were sampled on day 12. The probability of

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nest failure did not differ across treatments (Fisher's exact tests; recapture: $p = 0.75$, day 12: $p = 0.40$).

Experimental females were targeted for capture and sampling (physiology and morphology) twice during the reproductive season. The first female capture occurred immediately before or during the incubation period, prior to experimental manipulations ($n = 60$), and the second capture occurred when nestlings were 6–11 days old (no less than 36 h after the behavioral observations described below; $n = 32$). Both female captures were conducted within a specified time window to limit the influence of circadian effects (2100 h–0100 h). Initial blood samples were collected from adults within 3 min of approach to assess baseline corticosterone levels (Romero and Reed, 2005), antioxidant capacity, and plasma oxidative damage, and again after 15 min of restraint to measure stress-induced corticosterone. Although males were also targeted for sampling during the same time window, because they are more difficult to capture we were not able to obtain repeated samples from enough males to enable a similar comparison ($n = 39$ during early reproduction; $n = 9$ during the nestling provisioning period). Nestling body mass ($n = 193$) was measured with a Pesola spring balance on days 1, 5, and 12 post-hatch. Both baseline and stress-induced blood samples were collected from nestlings on day 12. In nestlings, baseline samples were only included in analyses if they were collected within 2 min of nest disturbance as corticosterone levels appear to rise after this interval: when all samples (up to 3 min) were included, sampling latency is significantly associated with baseline corticosterone in nestlings ($F_{1,136} = 24.4$, $p < 0.0001$). All blood samples were collected from the brachial vein and placed on ice until they were returned to the laboratory. Plasma was separated from whole blood by centrifugation, and stored at −70 °C until analysis. Red blood cells were stored in lysis buffer at room temperature.

2.2. Maternal behavior

When nestlings were five days old, behavioral observations were conducted at all nests. The number of provisioning visits that each female made to the nest during a 30 min period between 0800 and 1300 h was recorded by observers that were trained prior to data collection to ensure consistency. Following the conclusion of the initial observation period, a model predator that represents a threat to both adults and nestlings was placed 1.5 (± 0.03) m from the nest. Two replicates of a model house cat (Wildlife Treasures, Ely, MN) were used for predator trials; this species was chosen because of the high rates of predation house cats impose on many songbird populations (Loss et al., 2013). Real house cats, as well as the predator models used here, induce rapid anti-predator behavior in nesting barn swallows (Vitousek et al., 2014). Observations then resumed for an additional 30 min period.

2.3. Hormone assays

Circulating corticosterone concentrations were assessed using an enzyme immunoassay (EIA) kit (Enzo Life Sciences, Plymouth Meeting, PA). Pre- and post-manipulation samples from each individual were assayed on the same plate, along with a six-standard curve. Assays were conducted in duplicate according to the instructions of the manufacturer. Prior to assaying experimental samples, assay procedures were optimized for barn swallow plasma as previously described (Jenkins et al., 2013). Briefly, plasma samples that had been stripped and spiked with a known amount of corticosterone were tested across multiple dilution values and concentrations of steroid displacement buffer (Wada et al., 2007, 2008). The optimal dilution factor was determined to be 1:40 with 2% steroid displacement buffer. Inter-assay variation was 9.6% and intra-assay variation was 10.9%, and the detection threshold was 0.30 ng/mL. Within-individual changes in circulating corticosterone levels were calculated for each female. Raw measured corticosterone concentrations (mean ± SE) were: pre-manipulation baseline: 6.3 ± 0.4 ng/mL, $n = 97$, pre-manipulation stress-induced: 36.5 ± 1.8 ng/mL, $n = 97$; post-manipulation baseline: 13.5 ± 1.2, $n = 41$, post-manipulation stress-induced 38.8 ± 2.7, $n = 41$, nestling day 12 baseline: mean ± SE; 3.3 ± 0.3 ng/mL, $n = 138$, nestling day 12 stress-induced: 26.5 ± 0.8 ng/mL, $n = 147$.

2.4. Antioxidant capacity and oxidative damage

Plasma collected during the initial sampling period (within 3 min of approach and disturbance) was used to assess within-individual change in plasma antioxidant capacity and oxidative damage in adult females. The OXY-adsorbent test (Diacron International, Grosseto, Italy) was used to assess the total plasma antioxidant barrier by quantifying the ability of plasma to resist oxidation by hypochlorous acid (HOCl). These methods have been described elsewhere (Costantini et al., 2011; Vitousek et al., 2013). Briefly, 5 μL samples (of 1:100 diluted plasma, reference standards, and blanks) were incubated with a 200 μL aliquot of HOCl solution for 10 min at 37 °C. Following incubation, 5 μL of chromogen solution (*N*,*N*-diethyl-*p*-phenylenediamine) was added, and the absorbance was read at a wavelength of 505 nm (BioTek Synergy HT; Vermont, USA). Measured values were calibrated with a reference standard, and are expressed in mM of HOCl neutralized per mL of sample. The intra-assay variability was 5.6% and inter-assay variability was 8.9%.

The concentration of reactive oxygen metabolites (ROMs) was determined using the d-ROMs test (Diacron International), which predominantly assesses hydroperoxides. In this test, metabolite concentration is determined colorimetrically following the reaction of metabolites with a chromogen mixture of alkyl-substituted aromatic amine. Briefly, plasma samples were added to 200 μL of acetate buffer mixed with 2 μL of chromogen (*N*,*N*-diethyl-*p*-phenylenediamine), and incubated for 75 min at 37 °C. Following incubation, the samples were centrifuged and 190 μL of the supernatant was transferred to a microwell plate so that the absorbance could be read immediately at a wavelength of 505 nm (Biotek Synergy HT). A reference standard was used to calibrate measured values and expressed in mM of H_2O_2 equivalents. For the d-ROMs test intra-assay variability was 7.9% and inter-assay variability was 6.7%.

2.5. Molecular sexing of nestlings

Sex was determined by amplifying 1 μl of genomic DNA from each nestling with highly conserved primers, P2 and P8, that anneal to exonic regions flanking the introns of the CHD-*W* and CHD-*Z* genes in a 10 μl polymerase chain reaction (PCR) (Griffiths et al., 1998). JumpStart *Taq* DNA polymerase (0.25 units, Sigma) was used with the following protocol: an initial denaturation step at 94 °C for 60 s, followed by 34 cycles of 94 °C for 45 s, 48 °C for 45 s, and 72 °C for 45 s, and 72 °C for 3 min for the final extension. PCR products were mixed with Sybr Green (Invitrogen) and loaded on a 3% agarose gel in 1x TAE buffer, and female-specific fragments were visualized with a bench top UV transilluminator digital imaging system (UVP, LLC).

2.6. Data analyses

The effects of brood size manipulations on the physiology and body mass of adult females and nestlings were tested using general linear mixed models (GLMMs) with a normal error distribution in SAS 9.3. Circulating corticosterone levels were natural log-transformed to meet assumptions of normality and homogeneity;

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models of within-individual changes in the stress response (the difference between baseline and stress-induced corticosterone) did not differ qualitatively from models of stress-induced corticosterone, so only the latter are presented here. All models included breeding site as a random effect and treatment (reduced, enlarged, control) and brood size manipulation date as fixed effects. Models of within-individual changes in female phenotype (physiology and body mass) also included elapsed days between captures as a fixed effect. All nestling models included nest identity as a random effect, and whether individuals were transferred or raised in their natal nest as a fixed effect. Nestling models with baseline corticosterone also included latency from initial disturbance to blood sampling as a fixed effect. Because it was not always possible to obtain all physiological measures from each individual, sample sizes differed slightly between models, and are reported in the text. To address whether brood size influenced parental investment we analyzed the relationship between treatment and the number of nestling provisioning visits made in the absence and presence of a model predator (overdispersed count data) using a generalized linear model with a negative binomial distribution and log link function. Models of provisioning rate in the absence of a model nest predator included treatment, date, and time of day. Models of provisioning rate in the presence of a model predator also included the distance from the model predator to the nest. The residuals of the models were examined using diagnostic plots to ensure normal distribution and homoscedasticity. Differences among treatment groups were analyzed using least-squares means. Parameter estimates are presented for all variables; in addition, least squares mean estimates are presented for categorical variables.

Repeatability in baseline and stress-induced corticosterone were calculated using linear mixed effects models (LMM) with 'rptR' (Nakagawa and Schielzeth, 2010) in R v.3.2.1 (R Core Team, 2015). The restricted maximum-likelihood method (REML) was used, with female identity as a random factor. Models were run with and without sampling period (early reproduction or nestling provisioning) as a fixed effect. Confidence intervals were estimated with parametric bootstrapping (1000 simulation iterations). Models that included sex or treatment as fixed effects did not differ from those presented here.

3. Results

3.1. The brood value hypothesis

Nests did not differ in brood size or total brood mass prior to manipulation (Brood size: treatment: parameter estimates (least squares mean estimates) control: 0.00 ± 0.00 (4.12 ± 0.21), reduced: 0.11 ± 0.27 (4.23 ± 0.19), enlarged: −0.13 ± 0.27 (3.99 ± 0.20), $F_{2,37} = 0.44$, $p = 0.65$, date: −0.03 ± 0.01, $F_{1,37} = 7.74$, $p = 0.009$. Brood mass: treatment: control: 0.00 ± 0.00 (9.53 ± 0.90), reduced: 0.03 ± 1.10 (9.56 ± 0.78), enlarged: −0.65 ± 1.09 (8.88 ± 0.83), $F_{2,36} = 0.29$, $p = 0.75$, date: −0.06 ± 0.04, $F_{1,36} = 3.25$, $p = 0.080$). The differences induced by the manipulations were apparent by nestling day five (Brood size: treatment: control: 0.00 ± 0.00 (4.01 ± 0.24), reduced: −1.86 ± 0.30 (2.15 ± 0.22), enlarged: 1.40 ± 0.30 (5.41 ± 0.23), $F_{2,37} = 71.41$, $p < 0.0001$, date: −0.02 ± 0.01, $F_{1,37} = 4.69$, $p = 0.037$, $n = 58$. Brood mass: treatment: control: 0.00 ± 0.00 (32.49 ± 2.63), reduced: −14.89 ± 3.28 (17.59 ± 2.35), enlarged: 6.47 ± 3.35 (38.95 ± 2.56), $F_{2,36} = 25.05$, $p < 0.0001$, date: −0.05 ± 0.11, $F_{1,36} = 0.28$, $p = 0.60$, $n = 57$), and persisted through the measurement period. On day twelve, enlarged nests contained significantly more nestlings and had higher brood mass than control nests, and reduced nests contained significantly fewer nestlings and had lower total brood mass compared to controls (Brood size: treatment: control: 0.00 ± 0.00 (3.12 ± 0.39), reduced: −1.22 ± 0.44 (1.90 ± 0.35), enlarged: 1.80 ± 0.44 (4.92 ± 0.38), $F_{2,37} = 29.69$, $p < 0.0001$, date: −0.04 ± 0.01, $F_{1,37} = 6.18$, $p = 0.018$, $n = 58$. Brood mass: treatment: control: 0.00 ± 0.00 (66.42 ± 6.35), reduced: −24.86 ± 7.26 (41.56 ± 5.76), enlarged: 25.08 ± 7.10 (91.50 ± 5.93), $F_{2,32} = 31.67$, $p < 0.0001$, date: −0.83 ± 0.24, $F_{1,32} = 11.71$, $p = 0.002$, $n = 52$).

Despite the changes in brood size induced by the manipulation, brood size treatment did not influence within-individual changes in baseline or stress-induced corticosterone in females (Table 1; Baseline (least squares mean estimates): control: 9.18 ± 2.06, reduced: 8.27 ± 1.79, enlarged: 7.81 ± 1.90. Stress-induced: control: 6.40 ± 5.84, reduced: 4.33 ± 5.14, enlarged: 1.94 ± 5.34). Similarly, changes in the body mass, antioxidant capacity, and plasma oxidative damage of females were unrelated to brood size

Table 1
Fixed effects from models of within-individual change in adult phenotype, and the phenotype of 12-day-old nestlings.

	Parameter estimate ± S.E.	*df*	*F*	*p*
Body mass ($n = 25$)				
Treatment	0.52 ± 0.90 (Reduced) 0.78 ± 0.86 (Enlarged)	2, 10	0.42	0.670
Manipulation date	0.05 ± 0.04	1, 10	2.04	0.184
Elapsed days	0.04 ± 0.03	1, 10	1.44	0.257
Baseline corticosterone ($n = 31$)				
Treatment	−0.91 ± 2.51 (Reduced) −1.37 ± 2.52 (Enlarged)	2, 14	0.15	0.864
Manipulation date	−0.05 ± 0.09	1, 14	0.36	0.561
Elapsed days	0.00 ± 0.07	1, 14	0.00	0.962
Stress-induced corticosterone ($n = 31$)				
Treatment	−2.07 ± 7.56 (Reduced) −4.5 ± 7.61 (Enlarged)	2, 14	0.18	0.840
Manipulation date	−0.28 ± 6.83	1, 14	1.00	0.333
Elapsed days	0.02 ± 0.21	1, 14	0.01	0.925
Antioxidant capacity ($n = 28$)				
Treatment	−5.46 ± 51.65 (Reduced) 47.78 ± 48.49 (Enlarged)	2, 12	0.73	0.501
Manipulation date	−3.21 ± 1.94	1, 12	2.74	0.124
Elapsed days	0.47 ± 1.50	1, 12	0.10	0.761
Oxidative damage ($n = 30$)				
Treatment	−0.04 ± 0.14 (Reduced) −0.17 ± 0.14 (Enlarged)	2, 14	0.90	0.431
Manipulation date	−0.01 ± 0.01	1, 14	2.18	0.162
Elapsed days	**0.01 ± 0.00**	**1, 14**	**5.10**	**0.041**

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manipulation treatment (Table 1; Body mass: control: −3.23 ± 0.62, reduced: −2.71 ± 0.57, enlarged: −2.44 ± 0.57; Antioxidant capacity: control: −13.00 ± 35.58, reduced: −18.36 ± 37.51, enlarged: 34.77 ± 32.26; Oxidative damage: control: 0.31 ± 0.11, reduced: 0.27 ± 0.10, enlarged: 0.14 ± 0.10).

Parental effort under standard conditions (measured as the number of trips to the nest during the initial observation period) was not associated with brood size treatment (Fig. 1a; treatment: control parameter estimate (least squares mean estimate): 0.00 ± 0.00 (1.71 ± 0.31), reduced: −0.41 ± 0.44 (1.30 ± 0.30), Wald $\chi^2 = 0.85$, $p = 0.36$, enlarged: −0.16 ± 0.42 (1.55 ± 0.25), Wald $\chi^2 = 0.15$, $p = 0.70$; date: 0.01 ± 0.02, Wald $\chi^2 = 0.21$, $p = 0.64$, time: 0.004 ± 0.002, Wald $\chi^2 = 3.17$, $p = 0.08$; $n = 36$). However, in the presence of a model predator, females breeding at enlarged nests made significantly more trips to the nest than control females, but not those at reduced nests (Fig. 1b; treatment: control: 0.00 ± 0.00 (−0.43 ± 0.60), reduced: 0.80 ± 0.76 (0.37 ± 0.50), Wald $\chi^2 = 1.12$, $p = 0.70$, enlarged: 1.67 ± 0.77 (1.24 ± 0.42), Wald $\chi^2 = 4.66$, $p = 0.031$; date: −0.003 ± 0.033, Wald $\chi^2 = 0.01$, $p = 0.94$, time: −0.003 ± 0.006, Wald $\chi^2 = 0.20$, $p = 0.66$, model distance: 0.04 ± 0.40, Wald $\chi^2 = 0.01$, $p = 0.92$, $n = 36$; least squares mean differences: control-enlarged: $z = 2.16$, $p = 0.031$, control-reduced: $z = 1.06$, $p = 0.289$, reduced-enlarged: $z = 1.26$, $p = 0.206$).

3.2. Brood size and nestling phenotype

Nestling body mass on day 12 was predicted by brood size treatment and sex (Table 2; Fig. 2a). Nestlings from reduced broods were heavier than those from control and enlarged broods (treatment least square means: reduced: 20.39 ± 0.81, control: 18.60 ± 0.81, enlarged: 18.04 ± 0.56; reduced-enlarged: $t = 3.33$, $df = 125$ $p = 0.001$, control-reduced: $t = 2.07$, $df = 125$, $p = 0.040$, control-enlarged: $t = -0.66$, $df = 125$, $p = 0.51$). Male nestlings were heavier than female nestlings (M: 19.51 ± 0.60, F: 18.51 ± 0.58; $t = 2.83$, $df = 125$, $p = 0.005$), There was no interaction between sex and treatment, and transferred and original nestlings did not differ in body mass. Nestling baseline corticosterone levels were significantly influenced by treatment (Table 2; Fig. 2b): offspring from reduced broods had lower baseline corticosterone than those from enlarged broods (treatment least square means: reduced: −0.14 ± 0.38, control: 0.65 ± 0.39, enlarged: 0.88 ± 0.30; reduced-enlarged: $t = -2.71$, $df = 36$, $p = 0.010$, control-reduced: $t = -1.91$, $df = 36$, $p = 0.064$, control-enlarged: $t = 0.52$, $df = 36$, $p = 0.61$). Sex, date, and transfer status were unrelated to baseline corticosterone. Nestling stress-induced corticosterone was not predicted by treatment, or any of the other variables in the model (Table 2; Fig. 2c).

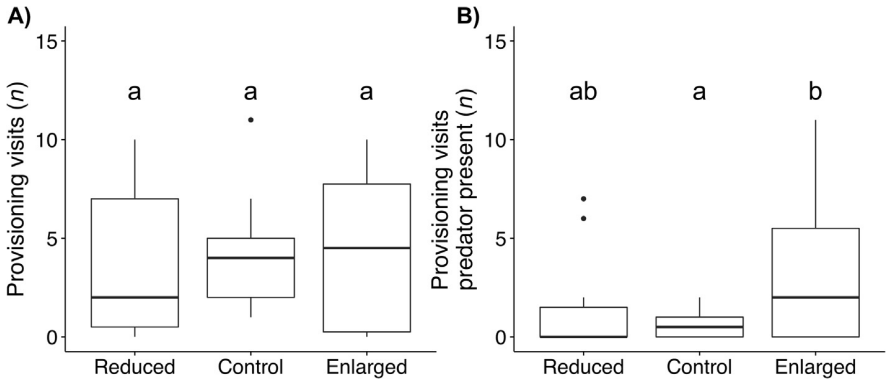

Fig. 1. Brood size treatment and offspring provisioning visits made by experimental females (a) under standard conditions, and (b) in the presence of a model predator. Box plots represent the median, 25th and 75th quartiles, and 95% CI. Significant differences are indicated by differing letters.

Table 2
Fixed effects from models of the phenotype of 12-day-old nestlings.

	Parameter estimate ± S.E.	*df*	*F*	*p*
Body mass ($n = 193$)				
Treatment	**2.29 ± 0.94 (Reduced)**	**2, 125**	**5.80**	**0.004**
	−0.75 ± 0.90 (Enlarged)			
Date	−0.04 ± 0.03	1, 125	2.10	0.150
Sex	**1.20 ± 0.68**	**1, 125**	**8.01**	**0.005**
Transfer	0.47 ± 0.71	1, 125	0.44	0.509
Sex*Treatment		2, 125	1.51	0.226
Baseline corticosterone ($n = 97$)				
Treatment	**−0.74 ± 0.48 (Reduced)**	**2, 36**	**4.07**	**0.026**
	0.43 ± 0.55 (Enlarged)			
Date	0.02 ± 0.01	1, 36	1.37	0.249
Sex	0.18 ± 0.46	1, 36	0.39	0.956
Latency	0.47 ± 0.27	1, 36	1.75	0.088
Transfer	−0.20 ± 0.43	1, 36	0.22	0.640
Sex*Treatment		2, 36	0.23	0.793
Stress-induced corticosterone ($n = 147$)				
Treatment	−0.16 ± 0.13 (Reduced)	2, 82	1.25	0.291
	−0.10 ± 0.14 (Enlarged)			
Date	−0.00 ± 0.00	1, 82	0.06	0.809
Sex	0.04 ± 0.09	1, 82	1.07	0.304
Transfer	−0.17 ± 0.11	1, 82	2.52	0.116
Sex*Treatment		2, 82	0.44	0.647

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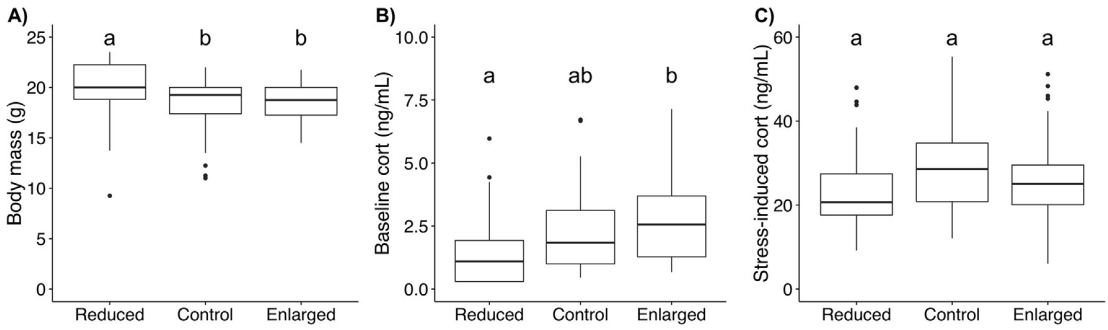

Fig. 2. Brood size treatment and nestling (a) body mass, (b) baseline corticosterone, and (c) stress-induced corticosterone at 12 days of age. Box plots represent the median, 25th and 75th quartiles, and 95% CI. Significant differences are indicated by differing letters.

3.3. Individual repeatability of hormonal responses

Baseline corticosterone was not individually repeatable in models that did not include sampling period (R(SE) = 0(0.11), 95% CI = [0, 0.35], p = 1), as circulating corticosterone increased in almost all individuals between the early reproductive and nestling provisioning periods. However, when sampling period was included as a fixed effect, baseline corticosterone was significantly repeatable (Fig. 3a; R(SE) = 0.48(0.14), 95% CI = [0.17, 0.73], p = 0.032). Stress-induced corticosterone was highly individually repeatable during the reproductive period regardless of whether or not sampling period was included in the models (Fig. 3b; R(SE) = 0.54 (0.13), 95% CI = [0.24, 0.76], p = 0.004; with sampling period as fixed effect: R(SE) = 0.57(0.13), 95% CI = [0.27, 0.77], p = 0.008).

4. Discussion

4.1. Brood value, glucocorticoids, and reproductive investment

Individuals often differ dramatically in their hormonal response to challenges. Yet it is not clear whether this variation reflects consistent individual differences in endocrine responses or their context-dependent flexibility, or is simply the product of variation in context alone. By experimentally manipulating brood size in barn swallows, we tested the hypothesis that individuals rearing larger, and therefore more valuable broods, rapidly suppress their glucocorticoid stress response to enable increased parental investment (Heidinger et al., 2006; Lendvai et al., 2007). While brood size manipulations induced changes in offspring number that persisted for the duration of the nestling period, maternal baseline and stress-induced corticosterone levels were unaffected by treatment. These results do not support the prediction that individuals modulate glucocorticoid responses based on shifting cues about potential reproductive gain (an extension of the brood value hypothesis: Lendvai et al., 2007). In order to determine whether this pattern reflects a general inflexibility in reproductive investment, or suggests that parental investment is modulated independently of glucocorticoid responses, it is necessary to examine how brood size treatment influences other aspects of parental phenotype (physiology, morphology, and behavior).

Overall, brood size manipulations had little impact on maternal physiology. Within-individual changes in female body mass, oxidative damage, and antioxidant capacity did not differ across treatments, suggesting that females at enlarged nests were not taking on greater costs. Likewise, provisioning rates in the absence of a model predator did not differ across treatments, despite marked differences in offspring number. However, in the presence of a model predator, females at enlarged nests maintained higher provisioning rates than those at control nests – although the provisioning behavior of females tending reduced broods did not differ significantly from the provisioning rate of birds in other treatment groups. The lack of a significant difference in provisioning rate between females rearing reduced broods and those in the other treatment groups in the presence of a model predator could be an artifact of the fairly low sample sizes obtained in this study, and the high variation in provisioning rate among nests in this treatment group. Increasing the number or duration of observation periods could also help to reduce the variation in provisioning rates that may be associated with changes in the intrinsic or extrinsic context of provisioning females.

Our results suggest that brood-value driven flexibility in parental investment is context-dependent. While swallows typically

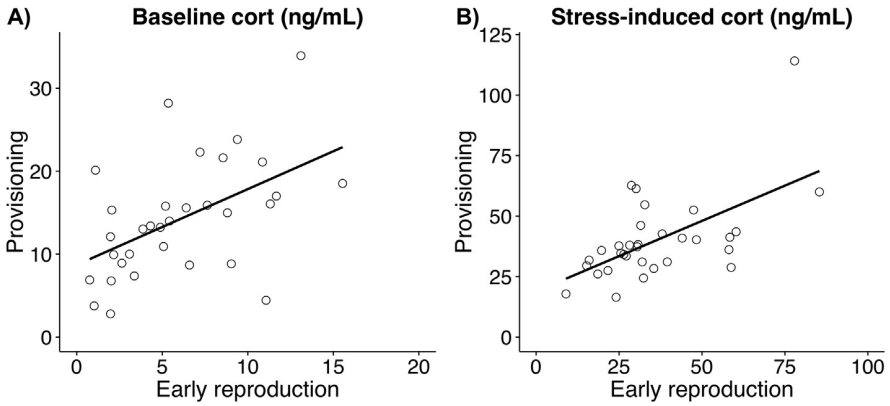

Fig. 3. Adult females' (a) baseline and (b) stress-induced corticosterone levels during the early reproductive and nestling provisioning periods.

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scale provisioning rate to brood size at natural nests, and often respond to brood enlargement by increasing provisioning rates under normal conditions (in the absence of a model predator), the magnitude of this effect differs across contexts and populations (Ardia, 2005; Costantini et al., 2014; Hainstock et al., 2010; Maguire and Safran, 2010). The lack of brood-size associated flexibility in parental behavior under normal conditions observed here could result from adverse conditions during the study period limiting females' ability to increase overall provisioning rates due to energy limitation. During our study, breeding conditions were particularly challenging: nest predation was high, and the weather was unusually variable. While barn swallows in this population typically produce two to three broods per season, in this season many individuals failed to fledge a single brood (Vitousek et al., 2014; Wildrick, 2012). When considered alone, the lack of a relationship between brood size and provisioning rate could also suggest that parents did not perceive enlarged broods as being of higher reproductive value, potentially as a result of context-dependency in the link between brood size and brood value (e.g., a larger brood may not have a greater chance of fledging offspring under particularly poor environmental conditions). However, while females at enlarged nests did not significantly increase overall provisioning rates, they appeared willing to take on greater risk to care for larger broods, making more trips to the nest in the presence of a model predator than females rearing control broods. This relationship suggests that enlarged broods are perceived as being of higher value than unmanipulated broods. Previous work in this population found that parents that mount a stronger corticosterone response to a standardized restraint stressor are more sensitive to the threat of predation, and provision offspring at lower rates under standard conditions (in the absence of a model predator; Vitousek et al., 2014). Taken together, these results indicate that individual variation in glucocorticoid responses can predict parental behavior, but that context-dependent flexibility in at least some aspects of reproductive investment is accomplished without concomitant shifts in glucocorticoid responses.

It is important to note that because of limitations in our experimental design, we cannot conclude with certainty that birds were perceiving and reacting to the model house cat as a predator. Immediately after the model was presented – which always occurred after the conclusion of the initial unmanipulated observation period – feeding rates declined rapidly (this study and Vitousek et al., 2014). In many cases, visit rates remained low to non-existent for the remainder of the observation period. It is possible that the birds did not perceive the model as a predator, but were reacting to the novelty of the object, or to some other factor in their environment. For this reason it has been recommended that, whenever possible, experimental studies of predation involve true replication, in which each individual is tested with an independent and unique stimulus (Johnson and Freeberg, 2016). True replication was not feasible in this study because of logistical constraints. Instead, we chose a study design with sacrificial pseudoreplication – in which more than one predator stimulus was used, but not enough to fully replicate the study. While preferable to simple pseudoreplication (Johnson and Freeberg, 2016), this approach still limits the ability to draw inferences about the generalizability of a response. However, regardless of how the model predator was perceived, our central finding is unchanged: that female barn swallows rearing larger broods demonstrate context-dependent shifts in parental investment that are not accompanied by changes in glucocorticoid regulation.

The two previous experimental tests of whether offspring number influences parental glucocorticoid responses – which used a similar experimental design to the one we utilized here – produced contrasting results. House sparrows provided with experimentally enlarged broods suppressed their glucocorticoid stress response (Lendvai et al., 2007), but smallmouth bass tending enlarged broods did not (O'Connor et al., 2011). However, while both of these studies assumed that parental investment was modulated based on brood size, neither study directly tested whether parental investment or offspring phenotype were influenced by brood size manipulations. When conditions are challenging, some aspects of reproductive investment (e.g., provisioning rate) may be maximal – particularly in short-lived species like songbirds – regardless of brood size. Future tests of whether individuals modulate glucocorticoid responses according to the brood value hypothesis should also include concurrent measurements of whether parental investment is flexibly modulated based on brood value.

While the brood value hypothesis makes explicit predictions about the modulation of the acute stress response, increased brood size could also elevate baseline corticosterone, either as an adaptive response to facilitate increased energetic demand (Bonier et al., 2009; Sapolsky et al., 2000) or as a reflection of the challenges associated with raising a large brood. Yet brood size manipulations have also yielded inconsistent effects on baseline corticosterone levels (Bonier et al., 2011; Hegner and Wingfield, 1987; Lendvai et al., 2007; O'Connor et al., 2011; Saino et al., 2002; Silverin, 1982). As described above, these contrasting results could be due in part to differences among species, populations, and even years in the extent to which aspects of reproductive investment are scaled to brood size. Alternatively, individuals of at least some species may be unable to rapidly modulate their glucocorticoid responses based on shifting cues about potential reproductive gain, either because of limitations in the ability to perceive or respond to these cues, or because modulating the glucocorticoid stress response does not facilitate increased reproductive investment.

4.2. Brood size and nestling phenotype

In contrast to the lack of brood size effects on parental body mass or corticosterone, brood size did influence nestling phenotype. As parents did not scale provisioning rates to offspring number in the absence of a model predator, nestlings reared in larger nests were likely chronically food-limited. Nestlings from reduced broods had significantly larger body mass than those from control or enlarged broods. Baseline corticosterone, but not stress-induced corticosterone, was also affected by treatment: nestlings from reduced broods had significantly lower baseline corticosterone than those from enlarged broods. The observed effects on baseline corticosterone are similar to those seen in response to a brood size manipulation in the European subspecies of barn swallow (*H.r. rustica*; Saino et al., 2003). However, these results contrast with the more typical pattern, whereby brood size treatment is not associated with nestling baseline corticosterone, even when manipulations alter body size or condition (Bize et al., 2010; Gil et al., 2008; Kozlowski and Ricklefs, 2011; Lobato et al., 2008). The relationships observed here could be due to the cumulative effects of high nest predation and unusually variable weather patterns at the study sites during the period of study (Wildrick, 2012) impacting the ability of parents to scale provisioning rates to brood size, and resulting in chronic and pronounced food-deprivation-induced stress in control and enlarged broods.

Our study, like the two previous tests of the glucocorticoid response to brood size (Lendvai et al., 2007; O'Connor et al., 2011), employed an unbalanced brood swap design. Nestlings were only transferred into enlarged nests; the remaining nestlings in reduced nests, and those in control nests, were handled but not transferred. In our study, this design resulted from the constraints associated with working in a small and relatively asynchronously hatching population. However, if the process of transfer is stressful to nestlings, or if parents perceive transferred nestlings as different

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from their own, it is possible that a subset of nestlings in the enlarged nests experienced a different environment than those in other nests. In this study, we found the greatest differences in phenotype between the reduced nests, and those in other treatments. This finding suggests that the presence of stressed or unrelated nestlings was not a primary factor driving differences in nestling phenotype among nests. Follow-up studies that use a balanced brood swap design – possibly over a longer time-scale – could provide greater confidence in these patterns.

4.3. Individual repeatability of hormonal responses

Baseline corticosterone, which increased between the early reproductive and nestling provisioning periods, was significantly repeatable only when sampling period was included as a fixed effect. Thus, while absolute levels of baseline corticosterone were not consistent, individuals with relatively higher baseline corticosterone levels during the early reproductive period also had relatively higher baseline corticosterone during the nestling provisioning period. Baseline corticosterone, which serves primarily as a metabolic hormone at low concentrations, often increases during demanding life history stages (Romero, 2002). The observed increase in baseline corticosterone from the early reproductive period to the nestling provisioning period is consistent with corticosterone promoting energy mobilization during demanding life history stages (Bonier et al., 2011; Crossin et al., 2012; Ouyang et al., 2013). Alternatively, baseline corticosterone may have increased because females were excessively challenged by the demands of reproducing in poor conditions, rather than as an adaptive response to increased energy needs (Bonier et al., 2009).

Stress-induced corticosterone levels were individually repeatable, regardless of whether sampling period was included in models. Stress-induced corticosterone levels, which are moderately heritable in this population (Jenkins et al., 2014), are an important predictor of reproductive success in female barn swallows (Vitousek et al., 2014). For example, we previously found that barn swallows with lower stress-induced corticosterone during the early reproductive period subsequently fledge more offspring when the risk of predation is high (Vitousek et al., 2014). This effect may be associated with individual differences in reproductive investment and the behavioral response to challenges. The significant individual repeatability of stress-induced corticosterone across the reproductive period seen in this study is consistent with the idea that reproductive strategy – at least in terms of overall energetic investment (e.g., provisioning rate in the absence of a model predator) – remained relatively fixed throughout the reproductive period, rather than responding flexibly to changes in brood size and thus potential reproductive gain.

The extent to which among-individual differences in circulating hormone levels result from consistent differences in endocrine function or flexibility, or reflect condition-dependent endocrine modulation, is not well understood. In addition to the potential for additive genetic differences to influence endocrine expression (Evans et al., 2006; Jenkins et al., 2013; Pottinger and Carrick, 1999; Satterlee and Johnson, 1988), endocrine traits can be permanently influenced by the developmental environment (Schmidt et al., 2014; Wada, 2014; Zimmer et al., 2013). Endocrine traits are also highly flexible and context-dependent, and can change rapidly in response to the social, physical, and physiological context of an organism (Lendvai et al., 2014; Taff and Vitousek, 2016). In practice, the endocrine response displayed by an individual represents the product of multiple hormonal reaction norms occurring across time-scales. Consistent individual differences in endocrine traits can therefore be difficult to discern, particularly in natural populations. The corticosterone repeatability seen here is consistent with findings of some degree of individual repeatability in glucocorticoid phenotypes in other species, particularly in stress-induced glucocorticoids (Cockrem et al., 2009; Cook et al., 2011; Vitousek and Romero, 2013; Grace and Anderson, 2014; Small and Schoech, 2015; but see Ouyang et al., 2011a). Because small changes in glucocorticoids influence metabolic regulation and support responses to changes in energetic availability and demand (Baugh et al., 2014; Ouyang et al., 2011b; Small and Schoech, 2015) it is not surprising that baseline glucocorticoid levels appear to have relatively low repeatability in free-living organisms. Moreover, individual differences in rapid glucocorticoid responses to environmental perturbations may conceal among-individual differences in baseline glucocorticoids (Lendvai et al., 2014; Taff and Vitousek, 2016). Analyses that take into account the changing demands within and across life history stages and environments can provide insight into the degree to which glucocorticoid phenotypes are consistent and heritable traits (Hau et al., 2016).

4.4. Conclusion

In contrast to the predictions of the brood value hypothesis, barn swallows breeding under somewhat challenging environmental conditions did not suppress the glucocorticoid stress response, or increase standard provisioning rates, when raising higher value broods. However, females rearing enlarged broods appeared to be less sensitive to threat, maintaining higher provisioning rates in the presence of a model predator. These results suggest that the rapid modulation of glucocorticoid levels to facilitate parental behavior may be context-dependent. Future studies that experimentally test the causal links between short-term elevations in glucocorticoids and parental investment, as well as the flexibility of these traits over a range of breeding conditions (including the relatively favorable environments that enable more flexible investment) will help to elucidate whether the rapid modulation of glucocorticoids is a widespread mechanism by which organisms match reproductive investment with potential gain. Likewise, studies that address the presence of brood-value driven flexibility in other endocrine mediators of parental behavior (e.g., prolactin: Angelier et al., 2016; gonadal hormones: Hau, 2007) can provide further insight into the mechanisms of flexibility in reproductive investment. In conclusion, our results provide stronger evidence of consistent differences between individuals in glucocorticoid responses, and of the effects of developmental stressor exposure on nestling phenotype, than of the rapid modulation of glucocorticoids based on potential reproductive gain.

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