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Author for correspondence:
Maren N. Vitousek
e-mail: mnv6@cornell.edu

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The lingering impact of stress: brief acute glucocorticoid exposure has sustained, dose-dependent effects on reproduction

Maren N. Vitousek[1,2], Conor C. Taff[1,2], Daniel R. Ardia[3], Jocelyn M. Stedman[1], Cedric Zimmer[1], Timothy C. Salzman[1,4] and David W. Winkler[1,2]

[1]Department of Ecology and Evolutionary Biology, Cornell University, Ithaca, NY 14853, USA
[2]Cornell Lab of Ornithology, Ithaca, NY 14850, USA
[3]Department of Biology, Franklin and Marshall College, Lancaster, PA 17604, USA
[4]Department of Biology, University of Kentucky, Lexington, KY 40506, USA

MNV, 0000-0003-2855-0832; CCT, 0000-0003-1497-7990

Acutely stressful experiences can have profound and persistent effects on phenotype. Across taxa, individuals differ remarkably in their susceptibility to stress. However, the mechanistic causes of enduring stress effects, and of individual differences in stress susceptibility, are poorly understood. Here, we tested whether brief, acute increases in glucocorticoid hormones have persistent effects on phenotype, and whether effects differ according to the magnitude or duration of elevation. We used a novel method to non-invasively manipulate hormone levels on short time scales: the application of corticosterone gel to a model egg secured in the nest. Free-living female tree swallows (*Tachycineta bicolor*) exposed to several brief corticosterone increases during incubation showed dose-dependent differences in behaviour throughout the reproductive period. Birds receiving treatments that simulated higher or longer acute stress responses later provisioned larger broods at lower rates; the resulting offspring were smaller in size. Treatment did not influence female body condition, oxidative stress, reproductive success or inter-annual survival, but exposed females maintained higher baseline corticosterone after treatments ceased. Overall, these results indicate that brief, acute elevations in glucocorticoids in adulthood can have long-term consequences. Furthermore, individuals that mount a greater or longer acute stress response may be more likely to experience lingering effects of stress.

1. Background

Human experience has long suggested that brief but acutely stressful experiences can have lingering effects on behaviour and performance. Survivors of severe trauma—war, terror attacks, natural disasters—sometimes experience long-term impairment following these events, and exhibit a wide range of phenotypic and epigenetic effects [1,2]. Few studies in other species have directly quantified the sustained effects of brief but severe challenges (e.g. minutes to hours) in adulthood, but emerging evidence suggests that perceived predation risk can have profound implications on physiology, behaviour and demography across taxa [3–6].

Glucocorticoid hormones are central coordinators of the response to challenges (e.g. predation, food limitation, thermal stress, social challenges). Under normal (baseline) conditions, glucocorticoids function primarily as metabolic regulators; in response to stressors, their rapid increase triggers widespread phenotypic and transcriptomic changes [7,8]. Chronic elevations in circulating glucocorticoids (days to weeks) can impose physiological and fitness costs [7,8]. Short-term elevations have well-documented short-term impacts on behaviour and physiology [8–10], but little is known about whether

brief, acute glucocorticoid responses—which may be common in the wild—can have sustained impacts.

Individuals differ remarkably in stress resilience—defined as the ability to recover quickly from challenges. Variation in HPA-axis function is thought to influence the susceptibility to chronic stress, but it is not known whether mounting a stronger or longer glucocorticoid response to a short-term stressor increases the likelihood of enduring phenotypic effects [11,12]. Some support for this possibility comes from findings that in humans, susceptibility to post-traumatic stress disorder is predicted by functional polymorphisms in genes associated with HPA activity, and by epigenetic changes in their regulatory regions [2,11]. In other taxa, natural variation in the glucocorticoid stress response has been linked with behaviour, not only in the immediate aftermath of a stressor [13–16], but also under normal (non-acutely or chronically stressed) conditions [17,18]. These patterns could simply reflect covariation between stress physiology and personality or life-history strategy, rather than direct causal effects of glucocorticoids. Phenotypic covariation could be underlain by genetic correlations, or result from developmental effects, or environment-driven phenotypic flexibility [18–20]. However, these patterns are also consistent with the possibility that differing glucocorticoid levels in response to transient stressors have differing degrees of carry-over effects on phenotype.

Here, we used a novel experimental approach to test whether mounting brief, acute glucocorticoid stress responses has sustained effects on behaviour, phenotype and fitness, and whether variation in the duration or magnitude of hormone elevation influences its subsequent impacts. Experimentally testing the effects of rapid hormonal responses has traditionally been challenging in free-living species; most existing methods of hormone manipulation alter circulating levels for days to weeks. We used a novel method to experimentally increase circulating hormone levels in free-living tree swallows (*Tachycineta bicolor*) by exposing incubating females to corticosterone (the primary avian glucocorticoid) dissolved in gel applied to a model egg secured in the nest. This technique enables the remote and non-invasive manipulation of hormone levels for short periods of time (approx. 90 min), with a high degree of control over the duration of the simulated response. By directly manipulating corticosterone rather than exposure to stressors, this approach also avoids the potential impact of individual variation in risk perception on the response to stress.

Tree swallows, like many species, show individual variation in the glucocorticoid stress response. The magnitude of the stress response is individually repeatable and heritable in the population used in this study [21,22], which is consistent with findings in other species [23,24]. Female tree swallows that mount a stronger hormonal response to a standardized restraint stressor suffer lower reproductive success—particularly under challenging conditions and when baseline corticosterone levels are high—but may have slightly higher annual survival [22]. Thus, we predicted that birds exposed to several brief increases in glucocorticoids would reduce their parental care and rear smaller young that were less likely to survive. Furthermore, we predicted that this effect would be dose-dependent, with females exposed to simulated acute stress responses of higher magnitude or longer duration showing more sustained treatment effects, reflected in a shift towards survival over reproduction. Alternatively, brief corticosterone exposures could have no effect, or only a short-term effect, on phenotype and components of fitness.

2. Material and methods

(a) Study site

Experiments were conducted in free-living tree swallows nesting in boxes at a long-term study site in Ithaca, NY, USA (42.503′ N, −76.437′ W), from May to July 2015. Nest contents are recorded every 1–2 days throughout the breeding season, except nestling days 14–20 (to avoid premature fledging).

(b) Glucocorticoid manipulation protocol and validation

Incubating females were exposed to corticosterone dissolved in 60 µl of dimethyl sulfoxide (DMSO) gel [9] and applied to a model egg secured in the nest (via a tether) the previous day. The brood patch of incubating birds is devoid of feathers, and the skin highly vascularized, which facilitates rapid uptake. On each treatment day, birds in all treatment groups received two doses of corticosterone, or of the vehicle (DMSO) alone, separated by 60 min (first dose: 'low' and 'long': 2 mg ml^{-1} corticosterone, 'high': 4 mg ml^{-1} corticosterone, 'vehicle control': DMSO only; second dose: 'long': 2 mg ml^{-1} corticosterone, 'low', 'high', 'vehicle control': DMSO only). Model eggs in the nests of control females were left undisturbed. Following treatment, birds returned rapidly to the nest (typically in less than 2 min), and resumed incubating. Visual examination after the target bird's departure revealed no apparent gel remaining on the model egg, or elsewhere in the nest ($n = 45$). The effect of this protocol on circulating hormone levels was determined by capturing and taking a blood sample from a group of birds used only for validation experiments 30, 90 or 180 min after the first dose was administered (and within 3 min of capture). Incubating females in this group were sampled once or twice per season ($n = 44$ females, 69 samples); in birds sampled twice, treatments were separated by at least 4 days.

(c) Experimental procedure

In a separate group of females ($n = 63$; control $n = 12$, vehicle control $n = 11$, low cort $n = 12$, high $n = 16$, long $n = 12$), we tested the effect of receiving multiple simulated stressors over a 5-day period. Females were randomly allocated into treatment groups at first capture (incubation day 6 or 7), and received the protocol described above once per day on incubation days 8–12, but were not recaptured during the treatment period. The time of dosing was randomized to prevent acclimation.

Females were recaptured after treatments (provisioning day 3–5; recaptured $n = 57$; control $n = 11$, vehicle control $n = 10$, low $n = 12$, high $n = 12$, long $n = 12$); when possible the social mates of study birds were also banded at this time. Nestlings were banded and individually sampled 12 days after hatching. Body mass, wing length and structural size (head–bill length) were recorded, and body condition (scaled body mass index [25]) was calculated from measurements of body mass and structural size for experimental females. Blood samples were taken within 3 min of disturbance, and again following 30 min of restraint in a cloth bag. In nestlings, the efficacy of negative feedback was measured by injecting dexamethasone, a synthetic glucocorticoid, immediately after the second blood sample event, and collecting a final blood sample 30 min later. The dose used (4.5 µl g^{-1} dexamethasone sodium phosphate 2 mg ml^{-1}) induces maximal negative feedback in nestling tree swallows (dosage comparisons are provided in the electronic supplementary material).

Fledging success was recorded to test treatment effects on offspring survival-to-fledging and female reproductive success

(measured as the total number of nestlings fledged). Inter-annual return rate was assessed the following year; during that year, all females with active nests on day 6–7 of incubation were successfully captured. Because breeding site fidelity is high (approx. 88% in females in this population [26]), return rate is a rough proxy for survival.

(d) Incubation behaviour

Incubation behaviour was assessed by measuring changes in nest temperature. The model egg used for corticosterone dosing also contained a thermocouple attached to a HOBO data logger (UX100-014M; Onset Computer Corporation). Temperatures were recorded every 10 s from installation (incubation day 4) through hatching (accuracy: $\pm 0.6^{\circ}$C, resolution: 0.02°C). Raw temperature data were processed using a custom script in R v. 3.3.3 (R Core Team, 2016) that used break points (a threshold change between successive readings of greater than or equal to 1.5°C) to calculate average daily and nightly (based on civil twilight times) incubation bout duration and inter-bout duration for approximately 29 500 bouts, and the percentage of time spent incubating.

(e) Activity and provisioning behaviour

The reproductive behaviour of experimental females and their mates was recorded using radio-frequency identification (RFID) devices at each nest (installed on incubation day 4) (Cellular Tracking Technologies; Rio Grande, NJ, USA) [27]. Passive integrated transponder tags—attached to leg bands fitted to adults at first capture—encode a unique digital identifier that is recorded, along with a time stamp, when birds pass through or perch on the antenna.

The raw data included 18 199 h of RFID records (greater than 1.75 million tag reads) from experimental birds. During incubation, when trips to the nest are a poor proxy for actual incubation behaviour because females incubate intermittently while at nests—and sometimes return to the box without incubating—we assessed behaviour by comparing the total number of RFID reads among females, which indicates daily time spent at the nest-box entrance. During the nestling provisioning period, when it is very rare for parents to enter the nest-box without feeding offspring [28], distinct visits were identified using nestling age-based thresholds to filter duplicate reads associated with the same visit from the dataset (as described in the electronic supplementary material). The final dataset contained a total of 177 542 provisioning visits. Hourly feeding rates were compared among birds.

(f) Glucocorticoids and oxidative stress

Steroids were extracted from plasma using a triple ethyl acetate extraction and corticosterone levels measured in duplicate using a commercial EIA kit (DetectX Corticosterone, Arbor Assays: K014-H5). Oxidative damage and antioxidant capacity were assessed using the d-ROMs and OXY-adsorbent tests (Diacron International, Grosseto, Italy). Details are provided in the electronic supplementary material.

(g) Data analysis

Data were analysed using R v. 3.3.3. The short-term impact of treatment on circulating corticosterone levels (protocol validation) was assessed using Kruskal–Wallis tests, with post hoc Dunn tests of significant differences. The effects of corticosterone exposure on the phenotype and survival probability of females and their offspring were assessed using an information theoretic approach, in which candidate models were ranked by corrected Akaike information criterion (AICc) scores, and their relative support evaluated using AICc scores and Akaike weights. The best fit model(s) within each candidate model set are reported in the text. Glucocorticoid data were natural log-transformed; prior to transformation, a constant was added to each value to make all greater than 1 (baseline + 1, stress and post-dexamethasone + 150).

Mixed effect models were used to test the effects of treatment on female behaviour and offspring phenotype. Female identity was included as a random effect. All models were fit with linear mixed models except for models of activity during incubation and provisioning, which were fit with generalized linear mixed models (GLMM) with a Poisson distribution. The effect of treatment on female phenotype and reproductive success (number of offspring fledged) was assessed using linear models; effects on nest abandonment, offspring survival from hatching to fledging and inter-annual return likelihood were assessed with logistic regressions. All candidate model sets included combinations of treatment, clutch initiation date, clutch or brood size, and interactions between treatment and clutch/brood size. Candidate models of female behaviour also included incubation day or nestling age, and its interaction with treatment. Because ambient temperatures impact food availability [29], analyses of behaviour recorded with RFIDs also included daily/hourly ambient temperatures (measured by the Northeast Regional Climate Center approx. 1 km from the study site). The mean temperatures from nestling day 7 (the approximate onset of thermoregulation) through day 12 (day of measurement) were included in models of offspring phenotype. Models of hourly provisioning behaviour also included quadratic terms for brood size, nestling age, hour and their interactions. To control for variation in provisioning by social mates, hourly male provisioning visits were also included in candidate models of female provisioning. To determine whether restricting analysis to the subset of nests at which males were RFID-tagged ($n = 45$; $n = 9$ control, $n = 7$ vehicle control, $n = 12$ low, $n = 9$ high, $n = 11$ long) impacted results, a second set of candidate models was run using all nests with female provisioning data ($n = 54$; $n = 11$ control, $n = 7$ vehicle control, $n = 12$ low, $n = 12$ high, $n = 12$ long) that did not control for male feeding rate. Analyses of post-treatment female phenotype included candidate models with pre-treatment values for each phenotypic trait; candidate model sets of abandonment and inter-annual return likelihood also included female body condition. Intercept-only models were included in all candidate model sets.

3. Results

(a) Protocol validation

The treatment protocols produced a brief, acute increase in circulating corticosterone that fell within the natural range of variation of the response to restraint (figure 1). The magnitude and duration of elevation differed across treatment groups (Kruskal–Wallis tests: 30 min: $\chi^2 = 12.9$, $p = 0.005$, 90 min $\chi^2 = 9.00$, $p = 0.029$, 180 min $\chi^2 = 6.2$, $p = 0.100$). In the low and high treatment groups, corticosterone levels were elevated 30 min after treatment (Dunn: low-control: $Z = 2.57$, $p = 0.030$; high-control: $Z = 3.15$, $p = 0.005$), but did not differ significantly from the vehicle control group (DMSO only) by 90 min post-treatment (low-control: $Z = 0.05$, $p = 1.00$, high-control: $Z = 1.45$, $p = 0.452$). The long group had elevated corticosterone levels 30 min ($Z = 2.42$, $p = 0.047$) and 90 min ($Z = 2.41$, $p = 0.048$) after the first dose, but the groups did not differ at 180 min.

(b) Experimental treatment

Prior to the experiment, treatment groups did not differ in clutch size, body condition or corticosterone levels

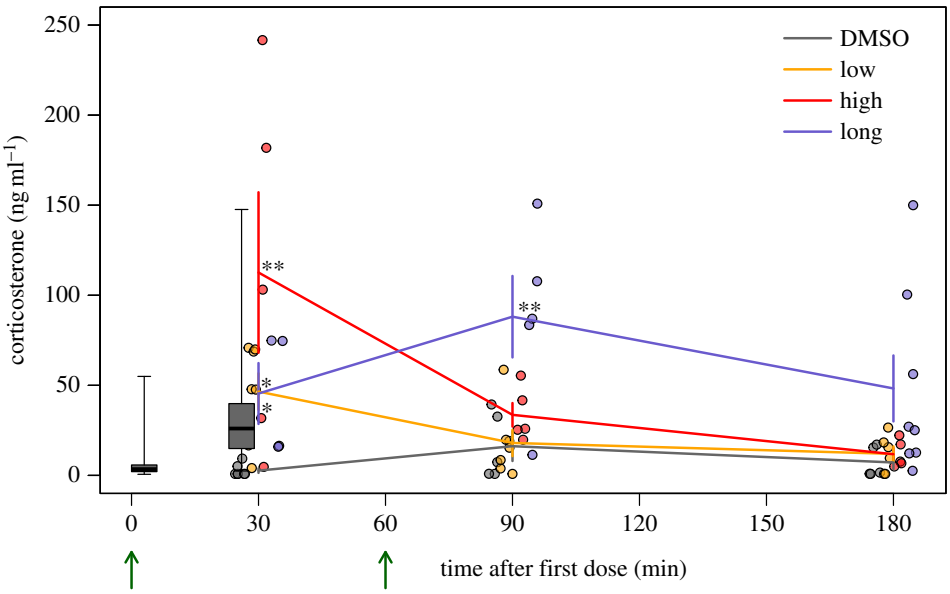

Figure 1. Immediate effects of corticosterone manipulation. Box-plots illustrate the mean and IQR and whiskers represent the range of natural variation in baseline ($n = 360$) and stress-induced corticosterone ($n = 366$; following 30 min of standardized restraint stress) in tree swallows in this population. Green arrows illustrate when doses (corticosterone + DMSO or DMSO only) were placed on model eggs. Coloured lines represent mean (±s.e.) corticosterone levels in birds sampled 30, 90 or 180 min after the first dose ($n = 69$); circles illustrate individual sample concentrations [30]. Asterisks represent significant differences between the control and treatment groups in post hoc tests (*$p < 0.05$, **$p < 0.01$).

(one-way ANOVAs; $p > 0.24$ for all tests). Exposure to brief, acute increases in corticosterone altered activity during incubation. Treatment was retained in both models of the daily time spent at the nest-box entrance during incubation that received strong support (table 1; electronic supplementary material, tables S3 and S4). Cort-treated females spent less time at the nest-box entrance than control birds; vehicle control birds were detected at intermediate rates (figure 2). The second-ranked model (ΔAICc: 1.1) contained the same main effects plus an interaction between treatment and clutch size (at larger clutches, there was a greater difference between the control and low cort, and control and vehicle control groups). Despite its impact on activity patterns, treatment did not alter incubation behaviour. Treatment was not retained in the best-fit models of daily or nightly per cent time incubating, incubation bout length or inter-bout interval. Treatment was also unrelated to the likelihood of nest failure during incubation (electronic supplementary material, tables S5 and S6). In each of these cases, the intercept-only model best fitted the data.

Offspring provisioning rates were significantly impacted by treatment. Best-fit models of female provisioning behaviour contained interactions between treatment and brood size, and between treatment and nestling age (table 1). Females in the high and long treatment groups rearing larger broods made fewer provisioning visits to the nest than those in other experimental groups, and increased provisioning rates less during the peak period of nestling growth (approx. days 6–14; figure 3). A similar analysis conducted using all experimental females (including those whose social mates were untagged) identified the same best-fit model and very similar treatment effects (table 1; electronic supplementary material, tables S7 and S8, and figure S2).

The offspring of corticosterone-exposed mothers were smaller than those from control nests. Maternal treatment, and the interaction between treatment and brood size, were retained in the best-fit models of nestling body mass and wing length (table 2 and figure 4; electronic supplementary material, table S9). For each of these measures, no alternative model that did not include treatment received strong support. Offspring head–bill length and corticosterone levels did not differ by maternal treatment (table 2). Treatment had no lasting impact on female body condition or on most metrics of female physiological state. Best-fit models of the body condition, antioxidant capacity, reactive oxygen metabolites and the corticosterone stress response of experimental females during the nestling provisioning period did not include treatment (electronic supplementary material, table S10). Baseline corticosterone levels were best explained by a model that included treatment and pre-treatment corticosterone; circulating levels were higher in treated birds (intercept = 0.96 ± 0.23, $t = 4.1$, $p < 0.001$; vehicle control = 0.33 ± 0.33, $t = 1.0$, $p = 0.32$; low = 1.37 ± 0.30, $t = 4.6$, $p < 0.001$; long = 2.01 ± 0.29, $t = 6.9$, $p < 0.001$; high = 2.09 ± 0.30, $t = 7.0$, $p < 0.001$; pre-treatment cort: 0.07 ± 0.03, $t = 2.2$, $p = 0.035$; pairwise comparisons in electronic supplementary material, table S11).

Neither offspring survival-to-fledging nor female reproductive success differed among groups (electronic supplementary material, tables S12 and S13). Half of all corticosterone-treated adults survived and returned to the site the following year (50% per group: 6 of 12 low, 8 of 16 high and 6 of 12 long); 17% of controls (2 of 12), and 18% of vehicle controls (2 of 11) returned. However, model comparison identified the intercept-only model as a better fit to data on inter-annual return than models that included treatment, or any of the other variables measured (electronic supplementary material, table S14).

4. Discussion

These results indicate that brief, acute increases in glucocorticoids can have sustained dose-dependent impacts on

Table 1. Candidate models of female behaviour. Linear mixed models include female identity as a random effect. Models that include brood size, nestling age, and hour, or their interactions with treatment also include their quadratic terms. All models within four ΔAICc of the best-fit model are shown, alongside the intercept-only model (in italics).

model	ΔAICc	log lik	*k*	weight
daily time at nest entrance during incubation				
treatment + clutch size + date + inc day + temp	0.00	−1653.6	11	0.54
treatment + clutch size + date + inc day + temp + treatment × clutch size	1.10	−1649.7	15	0.31
clutch size + date + incubation day + temp	2.78	−1659.4	7	0.13
intercept only	*196.8*	*−1760.6*	*3*	*0.00*
hourly nestling provisioning—nests with both parents tagged				
treatment + brood size + date + nestling age + hour + temp + treatment × age + treatment × brood size + brood size × age + male prov	0.00	−30 060.2	32	1.00
intercept only	*10 345.7*	*−35 263.2*	*2*	*0.00*
hourly nestling provisioning—all nests				
treatment + brood size + date + nestling age + hour + temp + treatment × age + treatment × brood size + brood size × age	0.00	−46 054.8	31	1.00
intercept only	13 029.7	*−52 598.8*	*2*	*0.00*

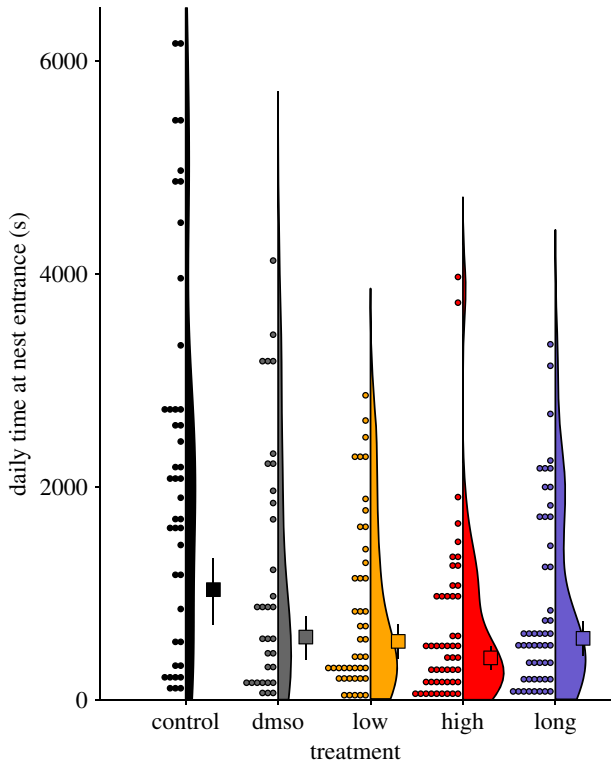

Figure 2. Daily time at nest entrance during incubation. Half violin plots show raw data on the left, and the model predicted mean and confidence interval for each treatment group on the right.

reproductive investment. Perceived risk can alter both reproductive investment and offspring phenotype [3–5]. Glucocorticoid hormones are often proposed to mediate this phenotypic flexibility, and several studies have experimentally linked perceived risk with subsequent changes in both glucocorticoid levels and reproductive investment [6,31]. However, it has been difficult to determine whether glucocorticoid responses alone are sufficient to induce these changes, or whether they also require the cognitive evaluation of threat or threat-induced changes in other phenotypic mediators (e.g. catecholamines, corticotropin-releasing hormone). Here, we used a novel experimental approach to separate the perception of a stressor from the endocrine stress response in free-living songbirds. Females exposed to increases in corticosterone similar to those triggered by transient stressors invested less in reproduction. Corticosterone-treated females rearing larger broods provisioned offspring at lower rates for the duration of the reproductive period—which ended weeks after transient hormone exposure had ceased. The offspring of corticosterone-exposed mothers had lower body mass and shorter wings than nestlings reared by control birds. These findings suggest that the hormonal responses triggered by transient stressors alone, independent of risk perception, can induce persistent changes in reproductive investment. Repeated exposure to transient stressors that only briefly activate the glucocorticoid stress response—such as temperature changes or anthropogenic or other disturbances—could therefore have longer-term impacts than was previously assumed.

Our results reveal that differences in stress resilience may be driven in part by variation in the rapid endocrine response to challenges. Individuals exposed to a simulated glucocorticoid response of greater magnitude (higher peak levels) or duration (slightly longer period of elevation) were more strongly impacted—both behaviourally and physiologically—than those that received a lower dose or control treatment. Therefore, transient stressors may have more prolonged impacts on individuals that naturally mount an endocrine response of greater magnitude, or those with weaker negative feedback. However, because HPA activity is mediated by many factors, including some downstream of hormone release (e.g. binding globulins, cofactors, receptor density and distribution), higher circulating hormone levels will not always equate to greater phenotypic or transcriptomic changes. Elucidating the effects of variation and covariation

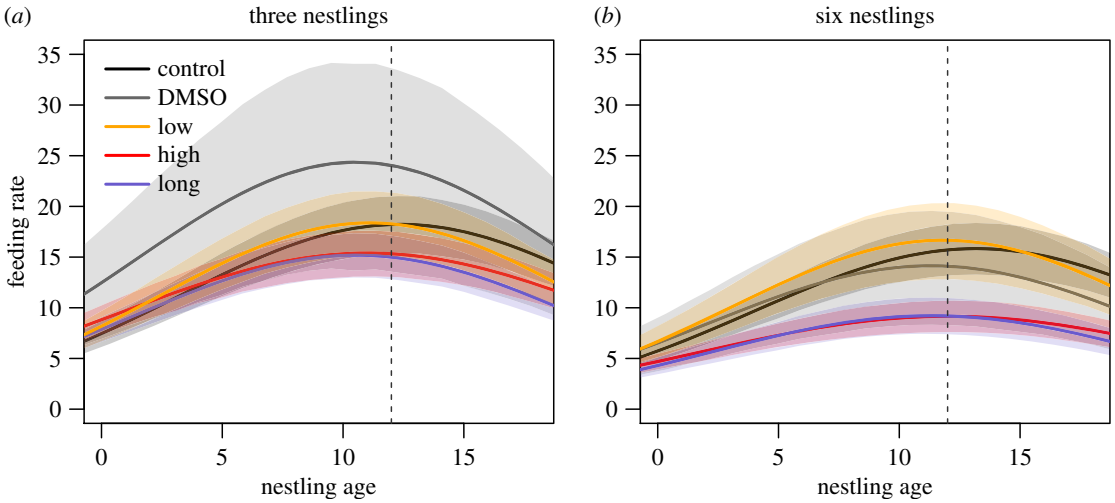

Figure 3. Effects of treatment on offspring provisioning rate. Curves show model predicted relationships ($n = 9391$ measures of hourly provisioning; 45 females). To illustrate the interaction between treatment and brood size, the relationship is plotted for broods with (*a*) three nestlings and (*b*) six nestlings. Hour, ambient temperature and clutch initiation date are held at their average values. The shaded region around each line represents the confidence interval. The vertical dashed line indicates the day of nestling phenotype measurement.

Table 2. Candidate models of offspring body size. Linear mixed models include nest identity as a random effect. All models within four ΔAICc of the best-fit model are shown, alongside the intercept-only model (in italics).

model	ΔAICc	log lik	*K*	weight
body mass				
temp + date + brood size + treatment + brood size × treatment	0.00	−495.6	14	0.84
intercept only	*7.37*	*−511.4*	*3*	*0.02*
wing length				
temp + date + brood size + treatment + brood size × treatment	0.00	−665.5	14	1.00
intercept only	*28.34*	*−691.7*	*3*	*0.00*
head–bill length				
intercept only	*0.00*	*−308.2*	*3*	*0.98*

in different components of this and other complex regulatory networks is important—if challenging—for future study [32,33]. However, the results of this study suggest that, at least in some contexts, variation in the endocrine response to transient stressors could carry-over to influence behaviour and reproductive investment under non-stressed conditions.

The effects of treatment on offspring provisioning rates were most pronounced in individuals rearing large broods, suggesting that brief glucocorticoid exposures may have context-dependent impacts on reproductive investment and fitness. Tree swallows, like many species, alter provisioning rates based on brood size and nestling demands [34,35]; however, the higher demands imposed by larger broods may force trade-offs between reproductive investment and self-maintenance [34,36]. If glucocorticoid treatment does induce a phenotypic cost, exposed females rearing larger broods may face different trade-offs between survival and offspring demand.

In addition to the sustained impact of glucocorticoids on provisioning behaviour, treatment also affected female activity during the incubation period. During incubation, corticosterone-treated birds spent less time at the nest entrance than control birds. These data alone do not reveal precisely what behaviours were impacted by treatment. Nest-box visits during the incubation period—unlike during nestling provisioning—do not necessarily provide information about reproductive effort, as females often make brief nest visits without incubating. Other behaviours, including surveillance—in which females perch in the entrance hole of the nest-box looking out—are also common during the incubation period, and are likely to be reflected in different patterns of tag reads. The lack of an effect of treatment on per cent time incubating, bout length or inter-bout interval suggests that treatment did not impact incubation; instead, differences in daily time at the nest-box entrance probably reflect treatment-induced changes in surveillance or other aspects of female behaviour.

Transient glucocorticoid exposures did not influence body condition, stress physiology or oxidative stress levels at recapture, or inter-annual return rates. These patterns suggest that experimental birds prioritized self-maintenance over reproductive investment. Females in the high and long treatment groups had elevated baseline corticosterone when measured one week after the conclusion of the treatment period (mean = 7.2 days, range 4–11). As the validation experiments confirmed that exogenous corticosterone was cleared rapidly (within approx. 90 min of exposure), this effect probably resulted from a treatment-induced change in HPA-axis regulation [37]. This longer-term, treatment-induced increase in

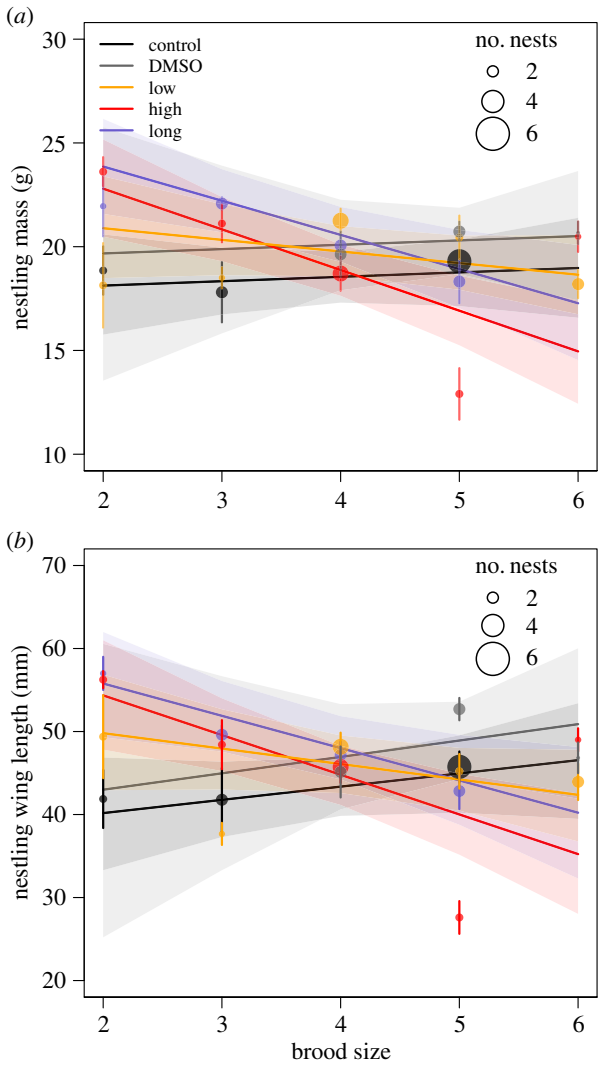

Figure 4. Effects of treatment on offspring phenotype. Lines illustrate model predicted relationships between brood size and (*a*) nestling body mass and (*b*) wing length across treatment groups ($n = 208$; control $n = 43$, vehicle control $n = 33$, low $n = 51$, high $n = 42$, long $n = 39$). Clutch initiation date and ambient temperature are held at their average values. Coloured circles represent the treatment mean and standard error for each brood size.

baseline hormone levels could be the proximate mechanism maintaining low provisioning rates in treated birds long after treatment had ceased. High glucocorticoid levels classically promote self-maintenance over reproduction [7,8]; however, experiments in which baseline glucocorticoids were manipulated over longer time periods have found variable effects on reproductive investment. In some cases, experimentally elevated glucocorticoids decrease offspring care; in other cases, elevation has no impact (e.g. [38–40]). Slight increases in glucocorticoids can even promote offspring provisioning, probably due to their role in promoting foraging behaviour and supporting energetically demanding behaviours [41,42]. However, post-treatment baseline levels in the 'long' and 'high' groups ('long': 26.9 ± 1.4, 'high': 40.9 ± 3.0 ng ml^{-1}, 'control': 3.0 ± 0.3) probably exceed the levels that promote reproductive investment; they are similar to those seen during particularly challenging environmental conditions in this population, which can be negatively associated with reproductive success [22].

The observed differences in parental behaviour were mirrored by changes in offspring size. Nestlings from large broods reared by mothers who had experienced greater or longer peaks in corticosterone had lower body mass and shorter wings than offspring from control treatments. Because nestlings were not measured after the mid to late provisioning period (day 12; to avoid premature fledging), we cannot determine if these phenotypic differences persist through the juvenile period or into adulthood, or if the nestlings of exposed mothers eventually catch up through compensatory growth. Nevertheless, these findings indicate that maternal experience significantly impacted offspring phenotype, at least temporarily. Although this experiment was designed to manipulate female corticosterone alone, and care was taken to ensure that the corticosterone gel contacted only the model egg, it is possible that the eggs of treated females took up some of the exogenous corticosterone. A pilot experiment in which the same concentration of gel was applied directly real eggs (avoiding the top to minimize the likelihood of female exposure) had no effect on nestling body size (GLMM with nest as random effect, $n = 68$: day 12 body mass: intercept: 19.2 ± 1.0, d.f. $= 13.4$, $t = 19.75$, $p < 0.001$, treatment: 0.12 ± 1.0, d.f. $= 13.4$, $t = 0.13$, $p = 0.90$, wing length: intercept: 40.7 ± 3.4, d.f. $= 14.0$, $t = 11.82$, $p < 0.001$, treatment: 1.3 ± 3.4, d.f. $= 14.0$, $t = 0.37$, $p = 0.72$; C.C.T. and M.N.V. 2016, unpublished data). Thus, it seems likely that the phenotypic differences in offspring observed in this study resulted from corticosterone-mediated changes in the behaviour of exposed females—which was observed both during incubation and provisioning—rather than from any potential direct effects of corticosterone exposure on offspring.

Despite differences in body size, treatment did not impact the likelihood that offspring would survive to fledging. This could be because the phenotypic shifts induced by treatment do not persist [43,44], or because under favourable conditions survival-to-fledging is only weakly dependent on offspring body size. It is also possible that transient stressor exposure has context-dependent effects on offspring survival that were masked by the mild environmental conditions during the period of study [29,45], or that these phenotypic changes represent adaptive responses to a challenging developmental environment [46]. We were not able to determine post-fledging survival rates in this study, but in general, tree swallows that fledge at lower body masses appear to have significantly reduced survival prospects over their first year [47,48]. The lower body masses of nestlings from cort-exposed mothers therefore suggest that they may suffer a survival cost post-fledging, particularly if the conditions they experience as juveniles do not match their developmental conditions [46]. Nestlings from larger broods showed more pronounced differences in body size by treatment than those from smaller broods. Overall, these patterns suggest that the potential for maternal stress exposure to have carry-over effects on offspring phenotype may be highly context-dependent, and that individuals from smaller broods may be better buffered from these effects.

Here, glucocorticoid levels were manipulated independently of perceived risk (e.g. of predation, food limitation, social stressors, etc.). Concomitant increases in perceived risk and glucocorticoids could have greater impacts than either stimulus alone. This could explain why some previous manipulations of perceived risk of predation found stronger impacts on reproductive success than those seen here [5,6]; however, phenotypic and demographic responses to

experimentally elevated predation risk also appear to be highly variable across studies [49,50]. Future experiments that directly compare the effects of brief glucocorticoid elevations, changes in perceived risk and both manipulations together (ideally in individuals with a normally functioning or pharmacologically blocked glucocorticoid response) could help to elucidate when and how brief challenges have lingering impacts.

The novel method of glucocorticoid exposure used in this study enables the remote and non-invasive manipulation of hormone levels on relatively fine temporal scales in free-living birds. Hormone implants, a widely used method of manipulation, alter circulating levels for much longer periods of time (days to weeks) [51,52]. Most available techniques for hormone manipulation also require animals to be captured to administer doses, which is itself a major stressor (e.g. implants, direct transdermal application) [37,38]. Several studies in free-living populations have manipulated hormone levels in adults by providing hormone-dosed supplementary food (e.g. [10,53]). This method can be used to manipulate circulating levels on shorter temporal scales; however, its use in free-living populations is limited to species or age classes that will reliably consume supplementary food, and forage in such a way that it is possible to ensure that the target individual alone consumes the dosed food item. The approach used here—in which incubating birds are topically exposed to hormones dissolved in DMSO presented on a model egg—could enable researchers working on a wider range of avian species to manipulate hormone levels on much finer temporal scales than has previously been possible. This method is, however, limited in its applicability across life-history stages, as it can be implemented only during incubation, and in the sex(es) that incubate. Nevertheless, we anticipate that the targeted use of this method could facilitate new insights into the short- and long-term impacts of hormones in natural systems. This technique could also be used to non-invasively deliver other compounds of interest to free-living birds (e.g. receptor agonists or antagonists, antibiotics, dietary supplements). It could potentially also be used to deliver prophylactic or therapeutic treatments in threatened populations.

5. Conclusion

These findings indicate that mounting several brief, acute glucocorticoid responses can have persistent effects on phenotype. Thus, lingering effects of acute stress, which are well documented in humans and increasingly supported in other taxa, may be driven at least in part by the immediate glucocorticoid stress response. Mounting a stronger or longer stress response appears to increase the likelihood of sustained impacts, potentially through its effect on subsequent HPA activity. In our validation experiment, birds that received a longer simulated response had peak hormone levels that were intermediate between the low and high treatment groups. Thus, we were not able to fully differentiate the effects of duration and magnitude. Nevertheless, the persistent effects in both of these treatment groups suggest that natural variation in glucocorticoid responses could causally affect stress susceptibility. Understanding how organisms are affected by the environments they experience has implications for a variety of biological disciplines, from integrative physiology to animal behaviour to community ecology to the evolution of life histories.

Ethics. All work was approved by Cornell University's Institutional Animal Care and Use Committee (no. 2001-0051).

Data accessibility. The datasets supporting this article are available in the Dryad Digital Repository (http://dx.doi.org/10.5061/dryad.s24121t). [30].

Authors' contributions. M.N.V. conceived of and designed the study. J.M.S. and M.N.V. carried out the study. D.R.A. and D.W.W. provided materials and support. C.C.T. and C.Z. conducted hormone assays and oxidative stress tests. T.C.S. implemented filtering thresholds for RFID data. C.C.T., D.R.A. and T.C.S. processed HOBO temperature data. C.C.T. and M.N.V. conducted statistical analyses. M.N.V. drafted the manuscript; all authors approved of the final version.

Competing interests. We declare we have no competing interests.

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