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Physiology

# Efficacy of negative feedback in the HPA axis predicts recovery from acute challenges

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The glucocorticoid stress response mediates a suite of physiological and behavioural changes that allow vertebrates to cope with transient stressors. Chronically elevated glucocorticoid levels are known to result in a variety of organismal costs, but relatively little is known about the downstream effects of mounting a series of brief, acute spikes in circulating glucocorticoids. Conceptual models of stress suggest that repeated acute stressors might produce 'wear-and-tear' on the stress-response system when encountered in sequence. We used a novel technique to experimentally induce acute corticosterone spikes on either three or six consecutive days in incubating tree swallows. Consistent with the 'wear-and-tear' hypothesis, we found that (i) a sequence of corticosterone spikes produced cumulative effects on corticosterone regulation, (ii) treatment frequency predicted the severity of consequences, and (iii) individual variation in the ability to terminate the stress response through negative feedback predicted the duration of physiological disruption in the group that experienced the most frequent challenges. Our results illustrate the importance of assessing multiple aspects of the hormonal stress response and have implications for understanding both individual and population resilience to repeated transient stressors.

## 1. Introduction

Mounting an appropriate response to transient stressors is critical in a dynamic environment [1–3]. One of the primary ways that vertebrates accomplish this response is by rapidly increasing glucocorticoids through the activation of the hypothalamic–pituitary–adrenal (HPA) axis, which coordinates a suite of behavioural and physiological changes that mitigate the effect of stressors [4,5]. However, glucocorticoids themselves can impose costs when they are elevated repeatedly or over a prolonged period [6,7]. Abundant data demonstrate that prolonged elevation can produce downstream costs, such as immunosuppression and senescence [8,9]. Transient stressors during development can also have long-term consequences [10,11], but much less is known about the downstream effects of mounting a series of acute stress responses in adulthood [12].

The Reactive Scope Model makes specific predictions about experiencing repeated acute stressors [3,13]. The basic model posits that a physiological mediator, such as glucocorticoids, fluctuates within a range of normal values to maintain homeostasis in response to predictable environmental changes. However, organisms can also elevate glucocorticoids briefly in response to unpredictable changes up to a threshold value over which homeostatic overload occurs and detrimental effects begin to accrue. When stressors are brief or widely spaced, each stress response may occur independently. However, a series of stressors experienced in sequence can produce 'wear-and-tear' on the stress-response system (similar to 'allostatic load': [14]) that results in a reduced threshold to enter homeostatic overload and an accumulation of

damage [3]. This hypothesis can be tested empirically by simulating a series of acute stress responses and collecting a time series of samples before and after manipulations.

Here, we used a novel technique to induce brief corticosterone spikes non-invasively in free-living tree swallows (*Tachycineta bicolor*) [12]. An initial experiment demonstrated that just five spikes during incubation produces sustained effects on baseline corticosterone, provisioning behaviour and nestling phenotypes [12]. We built on these results by measuring the stress response and negative feedback efficacy, varying the number of spikes, and collecting a time series of samples after treatments to assess the impact of corticosterone spikes on HPA axis regulation immediately after treatments ended and over subsequent weeks. Using these data, we tested three specific predictions of the wear-and-tear hypothesis: (i) a sequence of acute corticosterone spikes should have cumulative effects on HPA axis regulation, (ii) individuals that experience fewer spikes should recover normal HPA function faster than those experiencing more spikes, and (iii) individuals that recover faster from each individual spike—via more effective negative feedback—should spend less time in homeostatic overload and, therefore, experience fewer lingering effects on HPA regulation.

## 2. Methods

We studied tree swallows in Ithaca, NY, USA, from May to July 2016. Nest boxes were checked at least every other day beginning in early May to identify dates of clutch initiation [12]. Females were captured between 07.00 and 10.00 on day 3 or 7 of incubation (pre-treatment). At this capture, we collected blood samples to measure baseline (within 3 min of capture) and stress-induced corticosterone (30 min after capture). Immediately after collecting the stress-induced sample, birds were injected with dexamethasone (4.5 µl $g^{-1}$; Mylan® 4 mg $ml^{-1}$ dexamethasone sodium phosphate, product no.: NDC 67457-422-00) to stimulate negative feedback; a final blood sample was collected 30 min later.

Females were randomly entered into one of four treatment groups that included a full control (Control, $n = 12$), a vehicle control (Vehicle, $n = 9$), and two groups that received either 3 (Short-Cort, $n = 13$) or 6 (Long-Cort, $n = 9$) days of corticosterone manipulation. In all treatments except for the Control group, 60 µl of dimethyl-sulfoxide (DMSO) gel was placed on top of a model egg when females were not in the box at a randomized time each day for days 1–6 (Vehicle and Long-Cort) or days 3–5 (Short-Cort) after the pre-treatment capture (details in [12]). In the corticosterone groups, the gel was mixed with corticosterone (Sigma-Aldrich product no. 27840) to a concentration of 4 mg $ml^{-1}$ (equivalent to the 'High-Cort' group in [12]). This procedure results in a brief spike of corticosterone followed by a return to baseline within 90 min. The dose was designed to fall within the high end of the range of natural stress-induced corticosterone in response to a standard restraint protocol in our population (validation of method described in [12]). Females in the Vehicle group received six doses of DMSO gel only, while the Control group did not receive any doses.

Females were recaptured twice after treatments. The first recapture occurred on day 12 or 13 of incubation (usually 1 day after doses ended so that recovery time was the same for all groups). At this recapture, we took only a baseline blood sample. The second recapture occurred 6–8 days after hatching (9–11 days after treatments ended). At this recapture, we again took a full stress series to measure baseline, stress-induced and dexamethasone-induced corticosterone levels. We determined corticosterone concentration in plasma samples using an enzyme immunoassay kit (Arbor Assays: K014-H5) previously validated in tree swallows [15]. Inter-plate variation was 5.7% and intra-plate variation was 10.6%.

**Table 1.** Candidate models with ΔAICc < 4 for each response variable. All models with interactions also include the main effect of each predictor.

| candidate models | LL | *K* | ΔAICc | $w_i$ |
|---|---|---|---|---|
| 1st recapture baseline corticosterone | | | | |
| ~treatment + pre-stress | −176.9 | 6 | 0 | 0.36 |
| ~treatment | −178.4 | 5 | 0.2 | 0.32 |
| ~treatment × pre-stress | −173.5 | 9 | 2.4 | 0.11 |
| ~treatment + pre-base | −178.4 | 6 | 3.0 | 0.08 |
| ~treatment + pre-dex | −178.4 | 6 | 3.0 | 0.08 |
| 2nd recapture baseline corticosterone | | | | |
| ~treatment × pre-dex + pre-base | −115.1 | 10 | 0 | 0.53 |
| ~treatment + pre-base | −123.9 | 6 | 2.6 | 0.14 |
| ~treatment × pre-dex | −118.6 | 9 | 2.7 | 0.14 |
| ~treatment | −125.7 | 5 | 3.1 | 0.11 |
| 2nd recapture stress response | | | | |
| ~intercept only | −154.6 | 2 | 0 | 0.71 |
| ~pre-stress | −154.4 | 3 | 2.2 | 0.24 |
| 2nd recapture post-dexamethasone | | | | |
| ~treatment | −132.2 | 5 | 0 | 0.34 |
| ~treatment × pre-dex | −125.2 | 9 | 0.3 | 0.29 |
| ~treatment + pre-base | −131.5 | 6 | 1.7 | 0.15 |
| ~treatment + pre-dex | −131.5 | 6 | 1.7 | 0.14 |
| ~treatment + pre-stress | −132.2 | 6 | 3.1 | 0.07 |

### (a) Data analysis

We analysed the effect of treatments on post-treatment baseline corticosterone at the first and second recapture and on post-treatment stress response (stress-induced minus baseline corticosterone) and dexamethasone-induced corticosterone at the second recapture using general linear models. Candidate models were fit to each of the four response variables and compared by AICc values using the 'MuMIn' package in R [16]. Model sets for each response included an intercept only model along with 10 additional models that combined treatment group along with pre-treatment levels of baseline, stress response or dexamethasone-induced corticosterone levels. We present the results for models with ΔAICc values less than 4 (electronic supplementary material, table S1 details full model set). Analyses and figures were produced in R v. 3.3.3 (R Core Development Team, Vienna, Austria). Sample sizes varied between analyses when we had insufficient plasma to measure corticosterone or when nests failed before the final sampling interval.

## 3. Results

Pre-treatment corticosterone did not differ between treatment groups (electronic supplementary material, figure S1; one way ANOVAS; pre-treatment baseline corticosterone: $F_{3,39} = 2.02$, $p = 0.13$; pre-treatment stress response: $F_{3,39} = 2.02$, $p = 0.13$; pre-treatment dexamethasone-induced: $F_{3,39} = 1.80$, $p = 0.16$).

**Table 2.** Best-fit models for each response variable. Intercept only model for 2nd recapture stress response is not shown. All models with interactions also include the main effect of each predictor.

| predictor | estimate | s.e. | *F*-value | *p*-value |
|---|---|---|---|---|
| 1st recapture baseline ~ treatment + pre-stress; $w_i = 0.36$; $F_{4,36} = 6.9$, $p < 0.001$ | | | | |
| pre-treatment stress | 0.4 | 0.2 | 2.7 | 0.10 |
| treatment | | | 8.3 | <0.001 |
| Control | −8.8 | 8.8 | | |
| Vehicle | 8.6 | 9.4 | | |
| Long-Cort | 40.5 | 9.1 | | |
| Short-Cort | 29.1 | 7.9 | | |
| 1st recapture baseline ~ treatment; $w_i = 0.32$; $F_{3,37} = 8.0$, $p < 0.001$ | | | | |
| treatment | | | 8.0 | <0.001 |
| Control | 2.5 | 5.7 | | |
| Vehicle | 3.3 | 9.0 | | |
| Long-Cort | 36.9 | 9.0 | | |
| Short-Cort | 26.7 | 7.9 | | |
| 2nd recapture baseline ~ treatment × pre-dex + pre-base; $w_i = 0.53$, $F_{8,23} = 14.7$, $p < 0.001$ | | | | |
| treatment | | | 31.9 | <0.001 |
| Control | −7.7 | 14.8 | | |
| Vehicle | 6.2 | 23.0 | | |
| Long-Cort | 0.1 | 19.3 | | |
| Short-Cort | 27.9 | 17.7 | | |
| pre-treatment dex (Control) | 0.2 | 1.2 | 1.9 | 0.18 |
| treatment × pre-dex | | | 4.8 | <0.01 |
| Vehicle | −0.2 | 2.1 | | |
| Long-Cort | 4.5 | 1.7 | | |
| Short-Cort | −1.3 | 1.6 | | |
| pre-treatment baseline | 3.2 | 1.4 | 2.4 | 0.03 |
| 2nd recapture dexamethasone-induced ~ treatment; $w_i = 0.34$; $F_{3,27} = 6.3$, $p = 0.002$ | | | | |
| treatment | | | 6.3 | 0.002 |
| Control | 8.8 | 6.5 | | |
| Vehicle | 0.7 | 9.5 | | |
| Long-Cort | 38.9 | 9.9 | | |
| Short-Cort | 13.5 | 8.7 | | |
| 2nd recapture dexamethasone-induced ~ treatment × pre-dex; $w_i = 0.29$; $F_{7,23} =$ , $p < 0.001$ | | | | |
| treatment | | | 8.4 | <0.001 |
| Control | 10.9 | 39.2 | | |
| Vehicle | −6.7 | 48.1 | | |
| Long-Cort | 103.5 | 44.3 | | |
| Short-Cort | −4.2 | 42.2 | | |
| pre-treatment dex (Control) | −0.2 | 3.8 | 1.6 | 0.23 |
| treatment × pre-dex | | | 3.8 | 0.02 |
| Vehicle | 0.7 | 4.6 | | |
| Long-Cort | −6.0 | 4.2 | | |
| Short-Cort | 2.0 | 4.1 | | |

All nests were sampled at the first recapture, but some failed before the second recapture; the rate of nest failure did not differ by treatment group (failed nests for Control = 3, Vehicle = 2, Long-Cort = 2, Short-Cort = 3; $\chi^2 = 0.03$, $p = 0.99$). At the first recapture, only models that included either treatment and pre-treatment stress response ($w_i = 0.36$) or treatment alone ($w_i = 0.32$) received substantial support (table 1). There was a non-significant trend for individuals

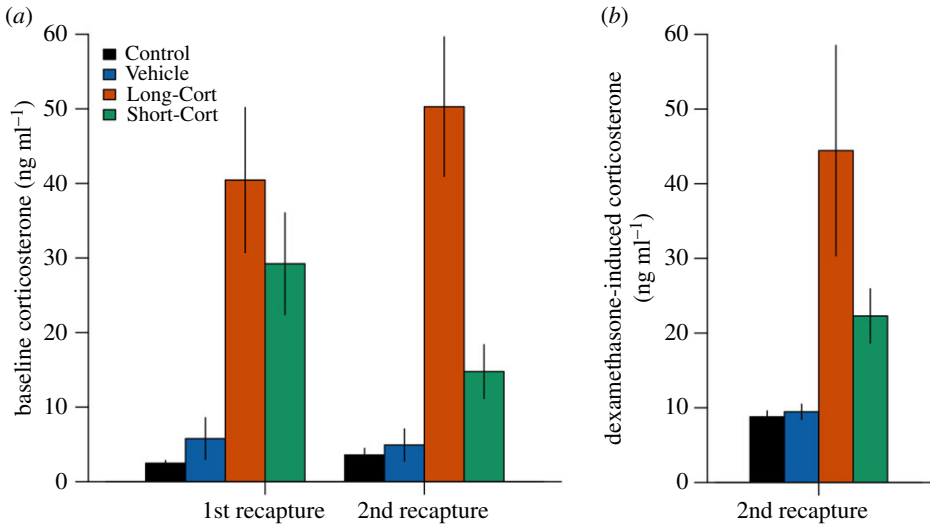


**Figure 1.** Main effect of treatments on (*a*) baseline corticosterone at 1st and 2nd recaptures and (*b*) post-dexamethasone corticosterone at 2nd recapture. Bars show mean ± s.e.m. from raw data.

with higher pre-treatment stress responses to have higher baseline corticosterone at the first recapture ($p = 0.10$; table 2). Baseline corticosterone was significantly elevated in the Short-Cort and Long-Cort groups compared to the two control groups, but did not differ significantly between Short-Cort and Long-Cort ($p < 0.001$, table 2, figure 1*a*; electronic supplementary material, figure S1).

At the second recapture, the best-fit model for baseline corticosterone included pre-treatment baseline corticosterone and an interaction between treatment and pre-treatment dexamethasone-induced corticosterone ($w_i = 0.53$). Baseline corticosterone was significantly higher in the Long-Cort group than in any other group and the other three treatments did not differ significantly from each other ($p < 0.001$; table 2, figure 1*a*). The significant interaction was driven by a positive relationship between baseline corticosterone at second recapture and pre-treatment dexamethasone-induced corticosterone in the Long-Cort group that was absent in the other three groups ($p < 0.01$; table 2, figure 2). Finally, pre-treatment baseline corticosterone was positively associated with baseline corticosterone at the 2nd recapture ($p = 0.03$; table 2).

No predictors were related to stress response at the second recapture (intercept only model $w_i = 0.71$; table 1). For dexamethasone-induced corticosterone at the second recapture, models that included either treatment alone ($w_i = 0.34$) or a treatment by pre-treatment dexamethasone-induced interaction ($w_i = 0.29$) both received substantial support (table 1). In both models, the Long-Cort group had significantly higher dexamethasone-induced corticosterone at the second recapture ($p < 0.001$; table 2, figure 1*b*). The interaction was driven by a negative relationship between pre-treatment dexamethasone-induced corticosterone and dexamethasone-induced corticosterone at the 2nd recapture in the Long-Cort group that was not present in the other three treatment groups ($p = 0.02$; table 2).

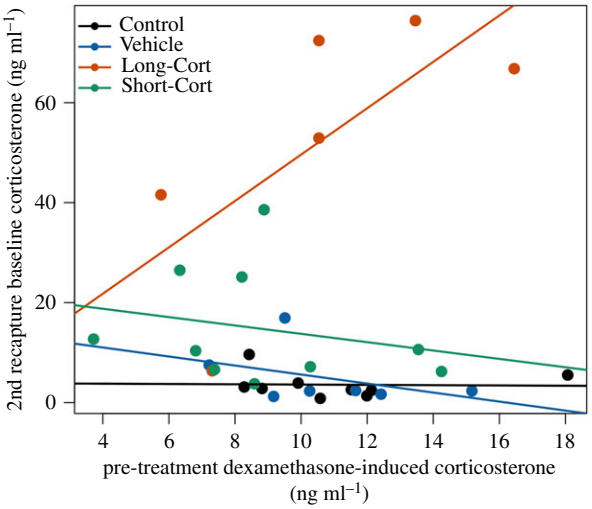


**Figure 2.** Interaction between dexamethasone-induced corticosterone and treatment in predicting baseline corticosterone at 2nd recapture. Best-fit lines are shown for illustration, but significance was assessed from the full model in table 2.

## 4. Discussion

We found that a few brief spikes in corticosterone during incubation produced sustained effects on HPA axis regulation that persisted well after treatments ended. Females that received three or six corticosterone doses during incubation had similarly elevated baseline corticosterone immediately after treatments. Recovery time differed between the groups, however, with the Long-Cort group having more sustained HPA disruption (i.e. slower recovery). Moreover, we found that variation in pre-treatment physiology predicted the lingering effects of corticosterone doses, but only in the Long-Cort group. In this group, HPA function in individuals with more effective negative feedback was less disrupted after experiencing a sequence of transient corticosterone spikes. These results are consistent with the 'wear-and-tear' hypothesis and suggest both that variation in the frequency of transient stressors influences HPA disruption and that between-individual variation in negative feedback predicts resilience to transient stressors.

Relatively little empirical work has focused on the efficacy of negative feedback, although conceptual models suggest that downregulation is important [3]. One previous study found that Galapagos marine iguanas (*Amblyrhynchus cristatus*) with more effective negative feedback were more

resilient to reduced food availability [17]. In our study, we were able to directly compare baseline, stress response and negative feedback corticosterone as predictors of resilience; only negative feedback was a strong predictor. Despite widespread interest in the stress physiology of natural populations, there is still little consensus about when glucocorticoids—or what aspect of HPA activity—reliably indicate past stress and little ability to predict between-individual variation in resilience [18]. Our results suggest that negative feedback is the best predictor of resilience and that both negative feedback and baseline glucocorticoids can be disrupted after a series of acute corticosterone spikes. More studies are needed that measure multiple aspects of the acute stress response before and after different stressors.

To date, most empirical work considers chronic and acute glucocorticoid elevation independently. Our results suggest that—at least in some cases—this categorization is not so clear-cut. When transient stressors are encountered repeatedly without adequate recovery time, a series of acute responses may result in prolonged modification of HPA axis regulation [1,3]. Combined with a previous experiment documenting altered parental investment and nestling phenotypes from a similar treatment [12], our results imply that a few brief stressors at critical times could result in relatively long-term costs similar to those documented in studies of chronic stress.

Ethics. Methods were approved by Cornell IACUC (protocol no. 2001-0051) and conducted with appropriate permits (permit no. 20576).

Data accessibility. Data are archived in the Dryad Digital Repository (http://dx.doi.org/10.5061/dryad.v00q39g) [19].

Authors' contributions. C.C.T., C.Z. and M.N.V. designed the study and collected data. C.C.T. and C.Z. assayed hormones. C.C.T. analysed data and wrote the manuscript with feedback from M.N.V. and C.Z. All authors approved the final version and agree to be held accountable for the content.

Competing interests. We declare we have no competing interests.

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