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Hormonal effects of maltreatment in Nazca booby nestlings: Implications for the "cycle of violence"

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ABSTRACT

Non-breeding Nazca booby adults exhibit an unusual and intense social attraction to non-familial conspecific nestlings. Non-parental Adult Visitors (NAVs) seek out and approach unguarded nestlings during daylight hours and display parental, aggressive, and/or sexual behavior. In a striking parallel to the "cycle of violence" of human biology, degree of victimization as a nestling is strongly correlated with frequency of future maltreatment behavior exhibited as an adult. Here, we investigate candidates for permanent organization of this behavior, including immediate and long-term changes in growth and circulating corticosterone and testosterone due to victimization, by protecting some nestlings with portable exclosures that prevented NAV visits and comparing them to controls. During maltreatment episodes, nestlings experience an approximately five-fold increase in corticosterone concentration, and corticosterone remains elevated approximately 2.8-fold until at least the following morning. Our results are consistent with the possibility that repeated activation of the hypothalamic-pituitary-adrenal axis permanently organizes future adult maltreatment behavior. No effect on growth, acute or chronic changes in testosterone, or chronic corticosterone elevation was detected or appeared to be components of an organizational effect. This unusual behavior presents an opportunity to investigate neural, endocrine, and behavioral organization resulting from early social trauma that may be conserved across vertebrate classes.

Introduction

Intergenerational transmission of adult–young maltreatment behavior is a well-known concept in human (Fang and Corso, 2007; Heyman and Slep, 2002) and non-human primate biology (Maestripieri, 2005; Maestripieri et al., 2005). This behavioral phenomenon, sometimes referred to as the "cycle of violence", has also been described in a wild bird, the Nazca booby (*Sula granti*; Müller et al., 2008). Despite extensive documentation (Chapman and Scott, 2001; Widom, 1989), little is known about the mechanism underlying intergenerational transmission. Three hypotheses exist: (1) maltreatment early in life causes long-term neuroendocrine changes that underlie later maltreatment tendencies (de Bellis and Keshavan, 2003; Maestripieri et al., 2005; Teicher et al., 2003); (2) young acquire maltreatment behavior through observational learning (Chapman and Scott, 2001); and (3) maltreatment is a genetically heritable trait (Caspi et al., 2002; Kaufman et al., 2004; McCormack et al., 2009; Widom and Brzustowicz, 2006). Here, we focus on the first hypothesis, asking if maltreatment causes hormonal changes in Nazca booby nestlings.

Nazca boobies are monogamous, ground-nesting, colonial seabirds. In a given breeding season, a large fraction of the adult population either does not breed (mostly males, due to a male-biased adult sex ratio; Maness et al., 2007; Maness and Anderson, 2008), or tries and fails (Anderson et al., 2004). These non-breeders (only non-breeders exhibit the behavior) search the breeding colony during daylight hours for unguarded nestlings. Upon locating one, the adult moves purposefully and directly to the nestling and engages in affiliative, aggressive, and/or sexual behaviors (Fig. 1). Virtually all nestlings are visited by these Non-parental Adult Visitors (NAVs) at least once during the nestling period and are vulnerable from approximately 30 days of age, when foraging parents leave nestlings unguarded, until approximately 80 days, when nestlings are mature enough to repel NAVs (Anderson et al., 2004). NAV behavior is apparently unique as naturally occurring, non-lethal, ubiquitous maltreatment involving unrelated adults and young. Intense social interest in young drives the interaction, even when a nestling has been experimentally relocated to a site outside a breeding colony (Townsend et al., 2002).

In general, adult animals interact in three common contexts with conspecific young that are not their own offspring. First, adults may eat these young, obtaining nutritional benefits (reviewed in Ebensperger, 1998 and Hrdy, 1979). Second, adults may harass or kill unrelated young, thereby reducing future or immediate competition between

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Fig. 1. Non-parental Adult Visitor (NAV) exhibiting aggressive behavior. Note the open ground-nest generally consisting of a few pebbles within a shallow bowl-shaped scrape. Nesting occurs at high density along the rocky coast and away from vegetation, providing clear sightlines for detection of unguarded nestlings by NAVs and for observation of nestlings and NAVs by investigators.

these individuals and the adults themselves or the adult's young (in mammals: Digby, 1995; Künkele, 1992; Rasa, 1994; reviewed in Ebensperger, 1998; in birds: Ashbrook et al., 2008; Cadiou et al., 1994; Ramos, 2003; Zicus and Hennes, 1989). Third, adults may act affiliatively toward non-offspring young; usually these friendly interactions occur among relatives or other members of social groups, and kin-selected and social benefits accrue to the adult (Packer, 1980; Pierotti, 1982; reviewed by Kalmbach, 2006 and Riedman, 1982). Nazca booby NAV behavior departs from the first two contexts inasmuch as NAVs do not kill nestlings outright (Anderson et al., 2004), and from the third context because Nazca boobies do not form highly social, extended family groups and their behavior is not restricted to affiliative interactions.

Adults may also associate with young in two much rarer contexts: “altruistic” affiliative interactions and forced sexual activity. Altruistic behavior, when unexplained by kin or social benefits may benefit the adult by increasing parental experience (reviewed by Riedman, 1982), or is costly to the adult and is generally classified as a mistake, including misdirected parental behavior (Angelier et al., 2006) and misidentification of young (Beaulieu et al., 2009; Brown, 1998; reviewed by Kalmbach, 2006). Sexual associations between adults and young are usually initiated by adult males and interpreted as a non-adaptive byproduct of a flexible female-recognition template (Besnard et al., 2002; Ewen and Armstrong, 2002; Somers et al., 2007). In birds, both altruistic and sexual interactions with non-familial young are typically anomalous and occur at very low frequencies under disturbed circumstances. In contrast, most (80%) non-breeding adult Nazca boobies (both male and female) exhibit NAV behavior at least once in a given breeding season (Anderson et al., 2004); given this high frequency, the motivation and action represent a usual part of the behavioral repertoire.

In the case of Nazca boobies, no benefit to the perpetrating adult is apparent, and so the behavior seems to have no direct selective reward for the perpetrators (Porter, 2003). Nazca booby nestlings incur injury and other costs during NAV events, including lacerations leading to death via ectoparasitic blood-feeding by landbirds (Curry and Anderson, 1987). Nestlings often adopt the submissive “pelican posture” (Nelson, 1978) throughout a NAV visit, with the head inclined forward until the bill rests against the neck, and the dorsal surface of the bill pressed against the ground. This posture protects the eyes, but prevents thermoregulation by gular fluttering and could lead to overheating (Anderson et al., 2004). Thus, selection does not appear to reward nestlings involved in NAV events, either.

Instead, the behavior of adults is connected to their experience as a nestling in two types of violent event. Around 1/3 of fledglings are the survivors of obligate siblicide as hatchlings (Humphries et al., 2006), during which circulating testosterone (T) is transiently, but sharply, upregulated, while corticosterone (CORT) remains unchanged (Ferree et al., 2004). As adults, this minority of the population is much more likely to engage in NAV behavior than are those that were not survivor hatchlings of siblicide (Müller et al., 2008). In a striking parallel to the “cycle of violence” in human biology, an even stronger positive predictor of the frequency of adult NAV behavior is the degree of victimization by NAVs (determined by number and severity of NAV interactions) experienced as a nestling (Müller et al., in review). These circumstances cast NAV behavior at the ultimate level as an epiphenomenon of nestling experience, without an apparent direct selective reward.

Returning to the proximate level, two of the existing hypotheses for causation of the cycle of violence in human biology (see above) do not provide an adequate explanation of NAV behavior. Observational learning is an unlikely explanation, because all nestlings have frequent opportunities to observe NAV behavior in the open, dense breeding colony, but adults vary widely in NAV behavior (Anderson et al., 2004). A possible genetic basis for NAV behavior has not been evaluated, but it could provide only a partial explanation, given the strong support for the two aspects of nestling experience as a driver. However, available data are at least consistent with endocrine organizational effects. During NAV events, NAVs exhibit higher circulating CORT and lower T than do contemporaneous non-breeders not engaged on NAV behavior (Tarlow et al., 2003a). The strong positive correlation between siblicide experience and NAV behavior tendency implicates physiological responses accompanying siblicide in permanent organization of aggressive NAV behavior. Such responses could include the transient perinatal androgen surge during the act of siblicide, or the higher circulating baseline CORT concentration in young hatchlings of a two-egg brood compared to hatchlings of a one-egg brood (Tarlow et al., 2001). Comparative evidence from the facultatively siblicidal blue-footed booby (*S. nebouxii*) is broadly consistent with this suggestion. Blue-footed booby nestlings hatch with a lower circulating androgen and CORT concentration than do Nazcas, and as adults, display a lower frequency of and less aggression during NAV interactions (Müller et al., 2008; Tarlow et al., 2001). In a noteworthy parallel, the only other bird species outside of the Sulidae known to exhibit a similar interest by adults in nestlings is also obligately siblicidal (American white pelican (*Pelecanus erythrorhynchos*); Somers et al., 2007).

However, NAV victimization is a stronger predictor of future adult NAV perpetration than siblicide and here, we identify the endocrine changes, if any, that NAV events induce in nestlings. Noting the potential psychological, thermoregulatory, immune, and probably other costs of NAV events on nestlings, we hypothesize that these events induce a hypothalamic-pituitary-adrenal (HPA) axis stress response in nestlings that may produce long-term neuroendocrine changes, either by chronic alteration of baseline hormones or by repeated acute HPA activation. Additionally, the caloric cost of resisting NAVs may depress the growth of nestlings, which may indirectly organize NAV behavior. We investigate immediate and long-term changes in nestling growth and circulating CORT and T due to NAV victimization, protecting some nestlings with portable exclosures that prevented NAV visitations and comparing them to controls.

Methods

All research reported here was permitted under the regulations of the Wake Forest University Institutional Animal Care and Use Committee, and adheres to NIH standards for animal use in research.

Selection and grouping of nestlings

In March, 2008, we identified participants in this experiment by placing uniquely numbered leg bands on 66 nestling Nazca boobies in a portion of our study site at Punta Cevallos, Isla Española, Galápagos Islands (1°23′S, 89°37′W) called the “Study Area” (see Apanius et al., 2008). Flattened wing chord provides a reliable estimate of age throughout the nestling period (Anderson, 1990) and measurements taken two days before the start of our study indicated that these nestlings were 40–60 days of age and within the window of vulnerability (30–80 days of age) to NAVs (Anderson et al., 2004).

Our experimental design matched "triplets" of age-matched nestlings that varied in treatment. "Experimental" nestlings were protected from NAVs by individual portable exclosures (open-top, circular wire mesh fences, mean diameter: 1.89 m) placed around the nest (see Fig. 1 for nest-site description) during daylight hours when parents were absent and were removed immediately upon return of a parent. The nestlings' natural movement was not restricted by exclosures, because nestlings of this age rarely leave the nest area. "Control" nestlings were never protected by an exclosure and received natural exposure to NAVs and little close contact with human observers. "Fence-Control" nestlings received an exclosure momentarily whenever the exclosure of its matched Experimental nestling was installed or removed. This treatment was intended to control effects on nestlings of human approach and fence installation/removal.

Between March 7 and March 30, 2008, we established 15 triplets, each containing one Experimental, one Control, and one Fence-Control. Individuals within triplets were randomly assigned to a group, with the exception of two nestlings that were excluded from the Experimental group because difficult terrain prevented exclosure installation, and so were placed in the Control and Fence-Control groups. The largest wing chord difference within a triplet (16 mm) indicated an age difference of approximately 2.4 days (Apanius et al., 2008). One Experimental, one Fence-Control, and one Control nestling died during the experiment. Cause of death was likely starvation, determined by emaciated appearance and declining weight. The Control nestling was replaced with an age-matched nestling in the study area that had also been monitored daily (see below). Neither the Experimental nor the Fence-Control nestling was replaced, and the other nestlings in their triplet were removed from the experiment.

For assignment of nestling sex, a drop of whole blood obtained by brachial venipuncture was blotted onto a small paper tab in the field and stored in 70% ethanol. Upon return to the lab, sex was assigned according to a modified PCR protocol of Fridolfsson and Ellegren (1999). Details of DNA extraction, the PCR protocol, and validation of the technique are provided in Maness et al. (2007) and Apanius et al. (2008). Maness et al. (2007) demonstrated 100% accuracy of this technique for this species.

Behavioral observations

All nestlings (Experimental, Fence-Control, and Control) were monitored daily from sunrise to sunset (approximately 5:45 AM–6:15 PM) for interactions with non-parents and parental attendance patterns. During NAV interactions, we measured the duration of the event and the proportion of time the nestling spent in a submissive pelican posture, and recorded sex of the NAV by sex-specific vocalizations (Nelson, 1978). NAV interactions were categorized non-exclusively as aggressive, affiliative, and/or sexual, and severity of aggressive events was determined on a scale of 1–4, following the methods of Tarlow et al. (2003a):

1 — Gentle and very infrequent biting, no shaking or pulling out feathers, no wounds created
2 — Biting, no shaking or pulling out feathers, no open wounds
3 — Biting, infrequent shaking and pulling out feathers, sometimes open wounds
4 — Vigorous shaking, biting, and pulling out feathers, leading to large open wounds (extreme).

On the seventh, eighth, and ninth days of the experiment, time–activity budget data were collected via point sampling at ten-min intervals for six nestling triplets (two triplets/day) to investigate the effect of fencing on nestling behaviors. Observations were conducted over three continuous morning hours and four continuous afternoon hours, or until parents for all focal nestlings returned. Behaviors recorded included: preen, beg, walk, stand, lie, sleep, stretch, vocalize, and play.

Blood sampling

Chronic response

To detect any chronic endocrine effects of NAV interaction on nestlings, we collected three repeated measures blood samples of 1 cm^3 from all nestlings by brachial venipuncture with unheparinized tuberculin syringes over 20 days (see Table 1). Samples were taken between 4:00 and 6:00 AM, when circulating CORT levels are least affected by external events in this species (Tarlow et al., 2003b). Not all nestlings could be sampled within this time frame on the same day, so triplets were divided into two groups: Group A and Group B. Group A was sampled on days 0, 10, and 20, and Group B was sampled approximately 3 days later, on days 3, 14, and 23. We allowed 3 days to pass before the initial sampling of Group B to avoid a period of rain, which can cold-stress nestlings. The second sampling of Group B was delayed an additional 24 h, also due to rain. All samples used in analyses of CORT were taken within 3 min of initial approach to the nestling (mean ± SD = 1.77 ± 0.55 min, N = 186) to avoid handling induced CORT changes (Romero and Reed, 2005).

Acute response

We collected additional 1 cm^3 blood samples from unprotected nestlings within 15 min of the start of an aggressive NAV event (mean ± SD = 11.73 ± 2.15 min, N = 11; see Table 1) following the above methods, to determine the immediate hormonal effect of NAV victimization on nestlings. NAV events ranged in severity of aggression from 3–4 (frequency of interactions at each severity rank: 3–82%, 4–18%, N = 11) and in duration from 3–12 min (mean ± SD = 5.86 ± 2.98 min, N = 11). Three samples were excluded from this analysis because they were obtained during less severely aggressive events. Nestlings were sampled as quickly as possible following the end of an event, or in two cases, following the end of aggressive and commencement of affiliative NAV interaction, which was interrupted for sampling. Three of these samples were obtained from Control nestlings within the experiment, and the remaining 8 samples were from unprotected nestlings outside of the experiment, but within the "Study Area." A time-matched sample was taken from Experimental and Fence-Control nestlings of approximately the same wing chord (mean difference ± SD = 27.79 ± 14.26 mm, N = 11, suggesting an age difference of approximately 4.1 days). Wing chord measurements indicated that these nestlings were 43–78 days of age (mean ± SD = 66.79 ± 7.17 mm, N = 33) at the time of sampling. Samples from nestlings within the experiment were obtained 2–7 days following the last baseline sample (mean ± SD = 4.36 ± 1.95 days, N = 25). We detected no correlation between CORT and the number of days between these samples within Experimental and Fence-Control nestlings ($r = 0.7$, $p > 0.05$, $N = 22$).

Table 1
Summary of blood samples obtained from nestlings. "Category" refers to the name of the category of blood samples in the text.

Category of blood sample	# of nestlings	Samples/ nestling	Description
Chronic response	13 triplets	3	Repeated measures over 20 days, obtained from 4:00–6:00 AM.
Acute response	11 triplets	1	Within 15 min following onset of NAV event, obtained throughout daylight hours.
Timescale of response	15	1	11–23 h following an aggressive NAV event, obtained from 4:00–6:00 AM. Compared to last chronic response sample.

Timescale of response

We examined the timescale of the nestling hormonal response to NAV interaction with 1 cm^3 baseline blood samples obtained between 4:00 and 6:00 AM from nestlings involved in an aggressive NAV event within the preceding 24 h ("Timescale of response;" see Table 1). Events ranged in severity of aggression from 2–4 (frequency of interactions at each severity rank: 2–53%, 3–40%, 4–7%, N = 15). These were not the same nestlings sampled for an acute response to NAV victimization, but a separate subsample of the study population. Six of these samples were taken from Experimental nestlings after the experiment ended and all exclosures were permanently removed. The remaining nine samples were obtained during routine baseline blood sampling sessions (see "Chronic response") from Control or Fence-Control nestlings that had received an aggressive NAV event of ranks 2–4 in the preceding 24 h. Sampling occurred 11–23 h after the aggressive NAV event (mean ± SD = 14.86 ± 3.26 h, N = 15) and within 9 days of the last baseline sample (mean ± SD = 6.53 ± 3.23 days, N = 15). Three additional samples were obtained from nestlings immediately following aggressive NAV events of severity rank 1 and combined with the above samples to evaluate the effect of severity on CORT response.

Assay characteristics

All samples were allowed to clot for 2 h, after which serum was separated from the cellular fraction by centrifugation at 6000 rpm for 5 min and then preserved in 95% ethanol (Goymann et al., 2007). Preserved samples were maintained at ambient temperature in the field and room temperature in the lab until analysis.

CORT and T concentrations were measured by quantitative competitive enzyme immunoassay (Assay Designs, Cat. No. 900-097 and 901-065) following manufacturer's instructions and verified in-house for avian serum, after double extraction in dichloromethane and diethyl ether, respectively. For CORT extraction, 120 μl aliquots of serum in ethanol were dried under a nitrogen stream and resuspended in 200 μl assay buffer. Steroids were double extracted with 2 ml dichloromethane; each extraction was vortexed for 2 min three times over 1 h at 0, 30, and 60 min, and the supernatant was pipetted off and dried under a nitrogen stream, then resuspended in assay buffer. Recovery efficiency of extraction and enzyme immunoassay averaged 82.5%, using the supplied corticosterone standard diluted to 1600 pg/ml in stripped chicken serum and ethanol (mean ± SD = 82.5 ± 19.77%, N = 7). For T extraction, 200 μl aliquots of serum in ethanol were dried under a nitrogen stream and resuspended in 500 μl of 0.05 M boric acid buffer. Steroids were double extracted with 2.5 ml diethyl ether, mixed for 1 h, flash frozen in an alcohol bath, decanted, and the supernatant was dried under a nitrogen stream and resuspended in assay buffer. Recovery efficiency of extraction and enzyme immunoassay averaged 80.87% after removal of one outlier (mean ± SD = 80.87 ± 23.47%, N = 5), using the supplied testosterone standard diluted to 2000 pg/ml in stripped chicken serum and ethanol. For CORT enzyme immunoassay, the detection limit was 26.99 pg/ml and intra- and inter-assay coefficients of variation were 6.6% and 7.8%, respectively. For T enzyme immunoassay, the detection limit was 5.67 pg/ml and intra- and inter-assay coefficients of variation were 10.8% and 14.6%, respectively. Because the primary antibody for CORT and T assays did not cross react to a significant degree with other circulating steroids, all measures are termed "corticosterone" and "testosterone" measurements, respectively. Finally, both assays were validated for use in booby serum for accuracy, precision, cross reactivity, and parallelism in measurements.

Growth measurements

Wing chord (flattened), culmen, and weight of all Group A and Group B nestlings were measured between 4:00 and 9:30 AM on the day following sampling of Group B, resulting in three sets of measurements for all study nestlings over the period of the study.

Statistical tests

Non-parametric tests (Friedman ANOVA, Chi Square, and Wilcoxon Matched Pairs) were used for all statistical analysis of hormone data, except for repeated baseline and severity of aggression in timescale of response data, because the hormone measurements did not meet the statistical assumptions of normality and homogeneity of variances. Timescale of response and chronic response CORT and T data were natural log transformed, after which residuals approximated normality. Repeated Measures ANOVA was used to analyze the timescale of CORT response by severity of aggression and repeated baseline CORT and T data by treatment group and sex. Within Control nestlings, rate of NAV interaction was binned by quartile and its effect on baseline CORT and T was analyzed with Repeated Measures ANOVA. Growth data met parametric assumptions without transformation and were analyzed using Repeated Measures ANOVA by sex and treatment group. For behavioral analysis, we first performed a principal components analysis that resulted in four behavioral components, described below. Because visual inspection of component scores suggested departures from normality, scores were analyzed using the non-parametric Friedman ANOVA.

Results

We installed and removed exclosures for Experimental and Fence-Control nestlings between one and four times/day according to parental attendance (mean ± SD = 1.30 ± 0.56 installations/day, N = 262). Experimental nestlings spent approximately 10 h/day within exclosures (mean ± SD = 9.78 ± 2.71, N = 244).

Between the first and the third baseline blood sampling sessions, Experimental nestlings experienced dramatically fewer NAV events than did Control and Fence-Control nestlings (ANOVA: $F_{2,38} = 8.01$, $p = 0.001$, Tukey HSD: $p < 0.025$; Fig. 2), with only 42% of Experimental nestlings experiencing at least one NAV visit compared to 100% of Control nestlings and 85% of Fence-Control nestlings. NAV interactions with Experimental nestlings were brief and occurred in the short period between a parent's departure and fence installation, and in one instance when an Experimental nestling left the exclosure briefly by squeezing through a gap between the fence and a rock. No NAV succeeded in entering an exclosure. During daytime hours, a nestling

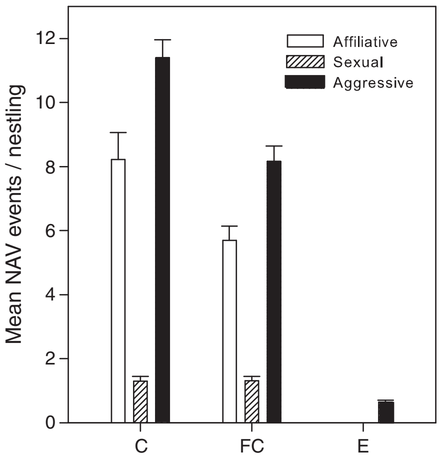

Fig. 2. Mean number and type of NAV events experienced by (C) Control, (FC) Fence-Control, and (E) Experimental nestlings. Vertical bars indicate standard errors. Mean severity of aggressive events ± SE was 1.52 ± 0.0054 for Control nestlings (N = 164), 1.42 ± 0.0080 for Fence-Control nestlings (N = 120), and 1.25 ± 0.052 for Experimental nestlings (N = 12).

attended by a parent was never visited by a NAV (966 nestling-day of observation). During nighttime hours, the nestlings were virtually always attended by a protective parent, and NAV events apparently do not occur at night (Anderson et al., 2004). Thus, exclosures nearly eliminated exposure of Experimental nestlings to NAVs during the period of the experiment.

Sex determination

Seventeen males and twenty-three females were divided among the three treatment groups. Two nestlings could not be sexed due to failure of DNA to amplify. These nestlings and their corresponding triplets were excluded from “Baseline across the study period” hormone analyses. We detected no sex differences in CORT or T concentrations, or in the growth parameters of wing, culmen, and weight (all $p > 0.5$); thus, we do not expect any direct influence of sex on our results.

Nestling behavior

Four principal components were derived from the nine observed behaviors: (PC1) preen/vocalize, (PC2) lie, (PC3) sleep, and (PC4) play. We observed no differences between treatment groups in any of the four components of nestling behavior (Kruskall–Wallis Test: PC1: $H_{2,18} = 0.082$, $p > 0.9$; PC2: $H_{2,18} = 3.52$, $p > 0.1$; PC3: $H_{2,18} = 0.047$, $p > 0.9$; PC4: $H_{2,18} = 0.14$, $p > 0.9$). Hence, fencing did not appear to affect nestling behavior.

Acute response

Nestlings experiencing an aggressive NAV event of ranks 3–4 had significantly higher CORT concentration than did contemporaneous age-matched Experimental (protected) and Fence-Control nestlings (Friedman ANOVA: $\chi^2_2 = 16.91$ $p = 0.0002$; Fig. 3A). One nestling triplet was excluded from T analysis due to insufficient serum, resulting in 10 nestling triplets analyzed for T. No difference was detected in T concentrations between treatment groups during a NAV event (Friedman ANOVA: $\chi^2_2 = 1.40$ $p > 0.4$; Fig. 3B).

Timescale of response

CORT concentration on the morning following an aggressive NAV event of severity ranks 2–4 was significantly higher than was the last baseline concentration (Wilcoxon Matched Pairs: $T = 6.0$, $N = 15$, $p = 0.002$; Fig. 4A). We found no differences in baseline T concentration following a NAV event compared to last baseline T concentration (Wilcoxon Matched Pairs: $T = 32.0$, $N = 14$, $p > 0.15$; Fig. 4B). One nestling was excluded from T analysis due to insufficient serum.

Nestlings that had experienced a NAV event of severity rank 1 exhibited significantly lower CORT concentration the morning following the event than those that had experienced an event of rank 2 (ANOVA: $F_{3,14} = 4.17$, $p = 0.026$, Tukey HSD: $p = 0.046$) or rank 4 (Tukey HSD: $p = 0.041$). CORT of nestlings that had experienced rank 3 events was not significantly different from that at any other rank (Tukey HSD: $p > 0.9$; Fig. 5A). We detected no differences in T concentration between severity ranks (ANOVA: $F_{3,13} = 1.71$, $p = 0.21$; Fig. 5B).

Chronic response

We detected no effect of treatment group, sex, or their interaction on baseline CORT and T concentrations across the study period (Repeated Measures ANOVA: $F_{4,54} = 2.05$, $p > 0.09$). We also detected no effect of rate of NAV interactions on CORT (Repeated Measures ANOVA: $F_{6,20} = 1.06$, $p > 0.4$) or T concentration (Repeated Measures ANOVA: $F_{6,20} = 0.92$, $p > 0.5$) within Control nestlings, across the study period.

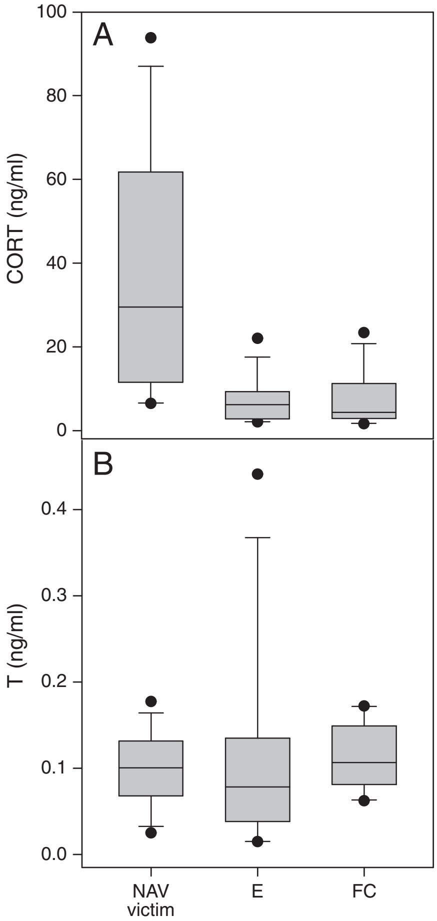

Fig. 3. Circulating concentrations of CORT (A) and T (B) of nestlings during or immediately following an aggressive NAV event (NAV victim) compared to time- and age-matched Experimental (E) and Fence-Control (FC) nestlings. Horizontal bars indicate means, boxes indicate 25th and 75th percentiles, vertical bars indicate 10th and 90th percentiles, and dots indicate outliers.

Baseline CORT concentrations increased across time and were significantly higher at the third sampling point than the first sampling point (Repeated Measures ANOVA: $F_{2,54} = 4.94$, $p = 0.01$; Tukey HSD: $p = 0.02$), consistent with a pre-fledging peak observed in other semi-altricial birds (Love et al., 2003). Baseline T concentration also exhibited a significant main effect of time (Repeated Measures ANOVA: $F_{2,54} = 5.79$, $p = 0.005$), as well as an interaction between time and sex: males at sampling point 3 had a significantly higher T concentration than at sampling point 2 (Repeated Measures ANOVA: $F_{2,54} = 3.66$, $p = 0.03$; Tukey HSD: $p = 0.008$). No other comparisons were significant ($p > 0.1$). No significant repeatability (r; Lessells and Boag, 1987) of CORT and T concentration was detected within nestlings, across the study period (CORT: $r = 0.002$, $p > 0.5$; T: $r = -0.14$, $p > 0.5$).

Growth

We found no effect of, or interaction with treatment and sex on the growth of wing, culmen, or weight across the study period (treatment effect on wing, Repeated Measures ANOVA: $F_{4,66} = 0.30$, $p > 0.8$; treatment effect on culmen, Repeated Measures ANOVA: $F_{4,66} = 0.1.67$, $p > 0.1$; treatment effect on weight, Repeated Measures ANOVA: $F_{4,66} = 2.21$, $p > 0.07$; all other effects, $p > 0.06$).

Nestlings were significantly underweight for their age (estimated by wing chord), compared to nestlings in the same area (“Study Area”) in 2002–03 (Repeated Measures ANOVA: $F_{1,36} = 41.43$, $p < 0.001$). During

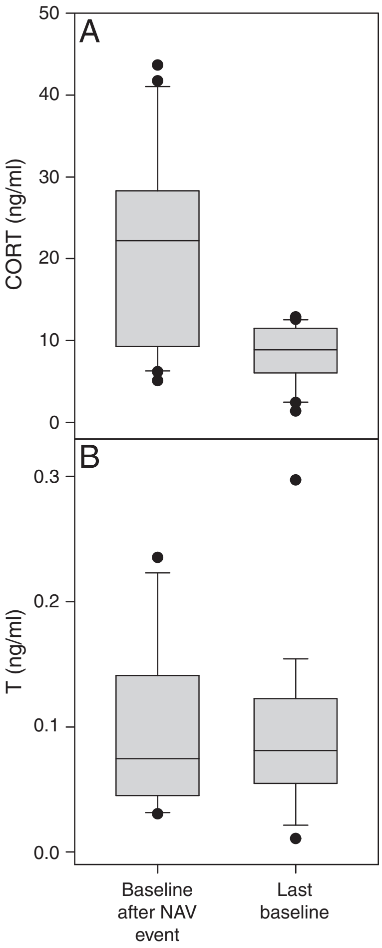

Fig. 4. Baseline circulating concentrations of CORT (A) and T (B) of nestlings the morning following an aggressive NAV event compared to that individual's last baseline sample. Horizontal lines indicate means, boxes indicate 25th and 75th percentiles, vertical bars indicate 10th and 90th percentiles, and dots indicate outliers.

the 2002–03 breeding season, 206 offspring fledged from the Study Area, well above the median of 143 fledglings (N = 15 years), while 67 nestlings fledged in the year of our study. These results indicate that the nestlings in our study were being raised under relatively poor environmental conditions, probably related to food delivery. Females were initially more underweight for their age than males were (Repeated Measures ANOVA: $F_{1,35}=7.95$, $p=0.007$, Tukey HSD: $p=0.02$). This effect disappeared by the third sampling session (Tukey HSD: $p=0.8$).

Discussion

This study establishes that NAV events induce a strong HPA axis stress response in nestlings, consistent with the possibility that this repeated activation of the HPA axis permanently organizes future adult NAV behavior. During victimization by NAVs, nestlings experience an approximately five-fold increase in CORT concentration compared to time- and age-matched Experimental and Fence-Control nestlings, and CORT remains elevated approximately 2.8-fold until at least the following morning. Over the longer term of several weeks, the treatment effect disappeared. Therefore, the induced driving of the HPA axis is a short-term, acute phenomenon.

Growth, acute or baseline T, and baseline CORT were not altered by treatment; neither were baseline CORT or T altered by rate of NAV interaction. As such, none of these appear to be affected sufficiently by NAV interaction to be regarded as potential effectors for a permanent organizational change, although we cannot rule out the possibility that baseline hormones may have been permanently altered by NAV victimization before the experiment began. However, we do not believe this to be the case because adult NAV behavior is predicted by degree of nestling victimization in a continuous, rather than qualitative manner (Müller et al., in review). We propose that the cumulative effect of a nestling's acute reactions contributes to the organizational effect that we described earlier (Müller et al., 2008).

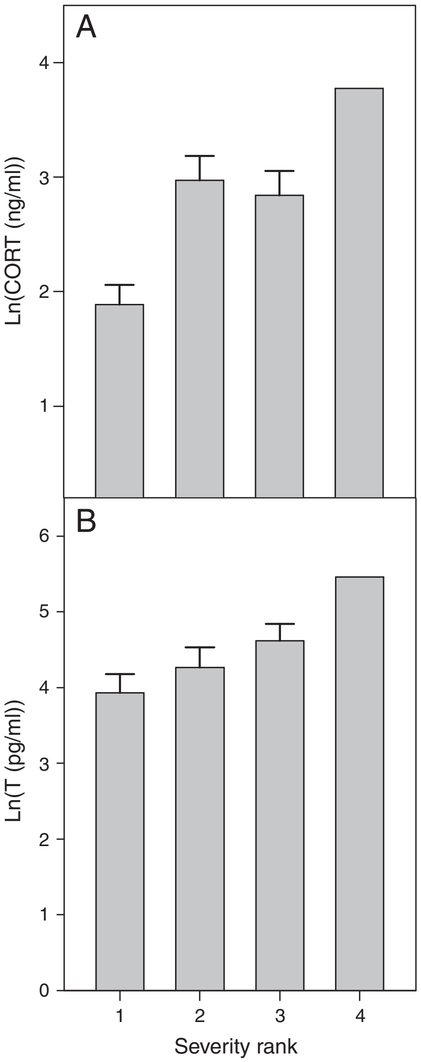

Fig. 5. Mean baseline circulating concentrations of CORT (A) and T (B) of nestlings the morning following an aggressive NAV event separated by severity of NAV event. Vertical bars indicate standard errors.

Support for an acute HPA response matches a broad pattern of results from mammals in which repeated short-term neonatal stressors cause no chronic stress response, but do result in long-term organizational impacts on hormones, brain, and behavior (Mirescu et al., 2004). Like Nazca booby nestlings victimized by NAVs, rat (*Rattus norvegicus*) pups isolated from their mother repeatedly displayed a rapid increase in CORT (four-fold after one hour of separation, slightly lower than the five-fold increase that we observed), but baseline CORT is unaffected (McCormick et al., 1998). Rat CORT concentration returns to baseline within 30–60 min of return to its homecage (McCormick et al., 1998), while Nazca booby CORT concentration remained significantly elevated after a NAV event until at least the following morning. Despite the ephemeral effect of maternal separation on neonatal rat CORT level, these repeated stressful events impair neurogenesis and cell proliferation in the dentate gyrus (Mirescu et al., 2004), causing permanent breakdown of negative feedback on CORT and increased anxiety-like behavior later in life (Boccia and Pedersen, 2001; Liu et al., 2000; Slotten et al., 2006; but see Huot et al., 2004 for a critique of the maternal separation paradigm). Neuroendocrine effects are generally more pronounced in females, likely because of sex-specific interaction between the HPA and hypothalamic-pituitary-gondadal (HPG) axis (Eklund and Arborelius, 2006; Li et al., 2004; Oomen et al., 2009), an interaction that may also contribute to an explanation of the more severe aggression that Tarlow et al. (2003a) documented in female Nazca booby NAVs.

Although we know little regarding long-term effects of repeated acute moderate-severe neonatal stressors in birds (see Jones, 1993, and Huff et al., 2001 for effects of neonatal handling, a mild stressor), such as those we observed, the long-term effects of chronic CORT elevation at the neonatal stage are strongly similar between birds and mammals. These effects include altered HPA axis stress response, and decreased neural development that results in compromised spatial and cognitive memory, smaller hippocampus with fewer neurons, and in songbirds a smaller high vocal center (in birds: Buchanan et al., 2004; Kitaysky et al., 2003; Pravosudov et al., 2005; Pravosudov and Kitaysky, 2006; Spencer et al., 2009; in mammals: see Sapolsky et al., 2000; Welberg and Seckl, 2001 for review). Female adult humans with a history of severe childhood physical and/or sexual abuse also exhibit permanent modification of hippocampal structure (Stein et al., 1997; Vythilingam et al., 2002).

Given the above similarities between the mammalian and avian systems, and the resemblance between CORT response to NAV victimization in Nazca booby nestlings and response to maternal separation in neonatal rats, we expect repeated NAV events to cause permanent neuroendocrine modifications similar to those exhibited by neonatal mammals under periodic stress. Modifications may include protracted HPA axis stress response, altered hippocampal structure, and increased anxiety-like behavior. Tarlow et al. (2003a) provided tentative support for an altered hormonal milieu in NAVs, which exhibit higher CORT and lower T during NAV events than contemporaneous non-NAVs do. Increased CORT may indicate anxiety, which is often associated with maltreatment tendency in humans and non-human primates (Maestripieri and Carroll, 1998) and may trigger or intensify NAV activities in the Nazca booby. However, it is currently unclear whether the altered hormonal milieu of NAVs is a stable characteristic, or induced by performing NAV behavior. Future work on this system could use a longitudinal approach (natal philopatry is essentially 100%; Huyvaert and Anderson, 2004) to determine whether the dynamics of the HPA axis in members of our treatment groups differ as adults in a manner consistent with a stable organizational effect.

Conclusions

Aggressive NAV events induce a strong acute HPA axis stress response in nestling Nazca boobies, similar to that observed in mammals. In mammals, such responses cause permanent neuroendocrine changes. If the same is true in Nazca boobies, these changes may increase individual propensity to perform NAV behavior as an adult and account for the observed intergenerational transmission of NAV behavior. Possible neuroendocrine modifications resulting from NAV victimization are a topic for later study.

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