Manipulative mothers

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Chapter 4

Experimentally simulating chronic stress in birds: a re-evaluation of corticosterone treatment methods

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Abstract

Short-term up-regulation of glucocorticoids in response to stressors is well-conserved and adaptive in vertebrates. However, prolonged exposure to high glucocorticoids is detrimental and in studying the effects of stress hormones, distinguishing short-term and long-term elevation is therefore indispensable. Since chronic stress in animals is becoming increasingly relevant due to rapid anthropogenic global change, a growing number of avian studies aim to test the fitness consequences of prolonged exposure to elevated corticosterone (CORT). Conventional hormone implantation methods, such as the Silastic capsule, induce a CORT profile with a high transient peak of plasma levels followed by a rapid return to baseline concentrations. Thus, in order to test the physiological consequences of long-term elevated CORT levels, more appropriate methods need to be applied. Therefore, we tested three alternative types of CORT administration in homing pigeons (*Columba livia domestica*): commercial pellets, self-made pellets and administration via food. Both types of implants resulted in a short-term peak in plasma CORT concentrations followed by a rapid drop to baseline. However, the food treatment succeeded in inducing relatively stable elevation for the entire four-week experiment. We suggest that only a pulsatile delivery of the hormone such as via food prevents a strong down-regulation of corticosteroid binding globulin (CBG) and ensuing accelerated hepatic hormone clearance in hormone-treated birds. Finally, while a decrease in egg production was related to the height of CORT exposure, an increase in body mass was related to the duration of CORT exposure, suggesting that CORT-mediated modulation of investment in reproduction vs. survival is rooted in different dimensions of the plasma CORT profile.

I. Introduction

Glucocorticoid up-regulation in response to stressors is a well-conserved phenomenon across the Vertebrata and appears to have various adaptive functions related to facilitating survival of life-threatening challenges. For example, elevated glucocorticoids mobilize energy stores (Sapolsky *et al.* 2000) and promote escape behaviour (Wingfield & Romero 2001), and channel energy away from reproduction towards self-maintenance via changes in behaviour and physiology (Greenberg & Wingfield 1987; Wingfield & Sapolsky 2003; Husak & Moore 2008). However, while short-term exposure to high glucocorticoid concentrations is extremely beneficial, prolonged exposure to high glucocorticoids actually harms individual health and accelerates aging (Sapolsky 1992; Sapolsky 1996; Sapolsky *et al.* 2000; Wingfield & Romero 2001) which explains why glucocorticoids typically increase only briefly and rapidly return to baseline levels (Greenberg & Wingfield 1987). In situations in which animals are unable to eliminate or escape the source of stress, however, the stressor and the accompanying elevation of glucocorticoids becomes chronic. Most studies implicitly define chronic stress as exposure to acute stressors at a high frequency for long period of time (Wingfield 2003; Rich & Romero 2005); the cumulative acute CORT responses results in overall higher CORT exposure. The potentially large costs of this chronic exposure to high glucocorticoids may under certain circumstances outweigh the benefits and become maladaptive (Sapolsky *et al.* 2000; Korte *et al.* 2005). In studying the effects of stress hormones, disentangling short-term and long-term elevation is therefore indispensable. However, in studies manipulating these hormone levels, the actual resulting plasma hormone profiles over time are rarely reported in detail.

The phenomenon of chronic stress is becoming more and more relevant as animals are faced with a rapidly increasing array of persistent anthropogenic stressors such as disturbance from tourism (Ellenberg *et al.* 2007; Thiel *et al.* 2008), increasing use of captive and wild animals in research (Taylor *et al.* 2008), and increased exposure to predators (Clinchy *et al.* 2004), noise (Wysocki *et al.* 2006) and pollution (Jenssen 2006) due to habitat fragmentation/degradation. In addition, climate change increases the frequency of extreme weather events (Kerr 2005) and also misaligns life history schedules (i.e. breeding, migration and moulting) with the phenology of seasons, which shift as the planet warms and rainfall patterns change (Wingfield 2008). While in the past the fitness benefits of the short-term, acute glucocorticoid stress response may have made selection against the costs of occasional prolonged exposure to glucocorticoids under chronic stress negligible, human-precipitated global change is increasing the incidence of chronic stress in vertebrates at a much more rapid time-scale than the rate at which evolution can create physiological solutions. The increased occurrence of chronic stress in animals may underlie the decline in reproduction and survival responsible for much of the

recent loss in biodiversity (Malcolm & Pitelka 2000; Walther *et al.* 2002).

While the nature of the acute stress response is well-studied (reviewed in Breuner *et al.* 2008), the endocrine dynamics associated with chronic stress are actually poorly defined in the literature. Due to a new awareness of the relevance of chronic stress for understanding changes in abundance and distributions of species, a growing number of experimental studies explore the physiological consequences and resulting fitness effects of prolonged exposure to elevated glucocorticoids in vertebrates. Many of these studies use birds as a model, and test the effects of artificially elevated corticosterone (the primary avian stress hormone) on various parameters related to survival and reproduction (Salvante & Williams 2003; Love *et al.* 2005; Burgeon & Raclot 2006). To properly test the detrimental effects of the prolonged corticosterone (CORT) exposure caused by chronic stress, it is necessary to experimentally simulate the CORT response pattern observed in birds under conditions of chronic stress in a physiologically realistic manner. The goal therefore is to produce a CORT profile showing a consistent pattern of hormone elevation within the physiological range for several weeks to months.

Until now, the conventional method of manipulating CORT involves subcutaneous implantation of a segment of Silastic tubing filled with crystalline hormone. For most steroid hormones, the Silastic capsule continuously releases a consistent dose of hormone over the course of weeks and generally results in long-term, relatively stable hormone elevation (testosterone: Buttemer & Astheimer 2000; Goerlich *et al.* Chapter 8; estradiol: Moore 1983). Using this procedure for CORT, however, results in a rapid peak in plasma CORT concentrations for a few days followed by a dramatic drop, often yielding lower plasma CORT levels than were recorded pre-implantation (Criscuolo *et al.* 2005; Angelier *et al.* 2007), despite the fact that the implants still contain hormone. Clearly, these very transient high concentrations inevitably followed by persistently low concentrations do not at all mimic natural CORT dynamics in birds experiencing chronic stress for weeks or months. To date, no consensus exists regarding an optimal method for elevating CORT in a biologically relevant manner for an extended period of time in birds.

In our study we test three alternative methods of CORT manipulation with the aim of inducing long-term elevation of plasma CORT levels. First, we tested two different CORT implants designed to release CORT in a continuous manner. One group of birds was implanted with a commercial cholesterol-based dissolving pellet, advertised to produce a sustained release of hormone as the pellet matrix degrades. These pellets have only recently been used in bird research (Bonier *et al.* 2007; Müller *et al.* 2009) and to date we lack detailed hormone profiles over an extended period of time. We implanted another group of birds with our own self-manufactured pellets using a method adapted from the fish literature (Duston & Pickering 1983): we mixed hardened palm fat with a known concentration of CORT, solidified it into a pellet form and implanted it subcutaneously in birds. This

method results in a sustained release of hormone within a physiological range for many weeks in fish (Gamperl *et al.* 1994), but has not yet been used in birds. Our third method of hormone treatment involved administering CORT in a less invasive and also more random pulsatile manner by mixing the hormone into the food. Because pigeons eat regularly throughout the day (but not during the night, Chabot & Menaker 1992), we expected this to be an effective way of simulating repeated exposure to stressors during daylight hours. This pulsatile elevation of hormone levels may be important because we hypothesized that in this way we could avoid the rapid decrease to basal levels due to active down-regulation of hormone concentrations in the circulation by the bird when receiving a continuous high rate of hormone input.

Our first objective was to test whether these three alternative methods of administering CORT mimic the prolonged, relatively consistent CORT elevation expected of animals experiencing chronic stress. Our second objective was to evaluate the consequences of both plasma CORT concentrations as well as duration of CORT elevation on reproductive output and self-maintenance. We tested the treatment methods on paired, breeding female homing pigeons which allowed us to assess the impact of chronic corticosterone exposure on egg-laying and maternal body condition. Finally, we integrate our findings with what is known about the dynamics of various components of the HPA-axis and discuss potential mechanisms underlying the CORT profiles resulting from different types of CORT treatments.

II. Methods

II-1 Animals and experimental design

This research was performed with permission from the Dutch Institutional Animal Care and Use Committee (DEC 4347E). We obtained the 78 (39 male and 39 female) homing pigeons used in this experiment from various pigeon breeders in the Netherlands. Females were assigned to one of three groups, each receiving a different method of CORT treatment (fat implant, pellet, food). Each treatment method group (hereafter referred to as "treatment") was further split into a control subgroup and 3 additional subgroups of increasing CORT dosage (low, intermediate, high). Females were housed indoors 3 weeks before the experiment started and were paired with males four days before we began CORT treatment (paired: 22 February 08; duration of experiment: 26 February 08 – 29 March 08). Birds in this experiment were housed in three rooms, with a light dark schedule of LD 14:10h (lights on: 0500, lights off 1900). All treatments and dosages were equally represented in each room. Birds received *ad libitum* mixed grain pigeon feed (Beyers Belgium), Supralith mineral supplement, grit and P40 pigeon grain (Kasper Faunafood).

II-2 Corticosterone manipulation

(a) Fat implants

The fat implant treatment was based on an approach commonly used in experiments using fish from the temperate zone, in which cortisol is suspended in melted cocoa butter and injected into the peritoneal cavity of the animals. After placing the fish into cool water, the cocoa butter hardens rapidly into a solid hormone implant. This method is highly effective in releasing stable cortisol rates in fish (reviewed in Gamperl *et al.* 1994) making it an attractive option for testing in birds. Given the considerably higher body temperature in birds (40°C) the approach required a fat with a much higher melting point than cocoa butter so that the fat would remain solid after implantation. We obtained hardened palm fat from Romi Foods (Herenveen, NL) which had a melting point of ca. 46°C. We hesitated to inject the fat intraperitoneally which would have required very high temperatures, so instead we created implants for subcutaneous insertion. Fat implants each contained 0.5 ml palm fat and 0, 5, 10, or 15 mg of CORT (for control, low, intermediate, and high subgroups, respectively). Dosages were selected to match dosages purchased for the dissolving commercial pellets (see below) and each dosage subgroup contained 3 birds (n = 12 fat-implanted birds). To create the implants we dissolved a known amount of crystalline CORT into pure ethanol, added a known quantity of melted palm fat, and heated the mixture until all of the ethanol had dissolved. We then pipetted the melted fat mixture into a plastic drinking straw (diameter of 0.5 cm) which was sealed on one end. After allowing the fat to harden, we sliced the straw into 2.5 cm lengths and slid the cylindrical fat pieces from the straw mould and stored them at 4oC until implantation.

(b) Pellets

Another group of birds were subcutaneously implanted with a commercial dissolvable pellet containing 0, 5, 10 or 15 mg of CORT in control, low, intermediate, and high dosage subgroups, respectively; each dosage subgroup contained 3 birds (n = 12 pellet-implanted birds). The self-degrading pellets are advertised (based on implantations in rodents) to release a continuous dose of hormone over 60 days and are comprised of mostly cholesterol as well as cellulose, lactose, phosphates, and stearates (Innovative Research, Sarasota FL, USA). We selected the dosages by scaling up the 0.1 mg CORT dosage used in Bonier *et al.* (2007) on white-crowned sparrows which typically weigh ca. 28 g, to the much larger ca. 400 g homing pigeon and then selected a lower and higher dosage.

(c) Food

The final treatment group received a daily serving of 50 g of pigeon feed mixed with 1 ml of vegetable oil containing quantities of dissolved crystalline CORT that yielded CORT concentrations of 0, 20, 30 and 40 mg CORT/ kg food in control, low,

intermediate, and high dosage subgroups, respectively. These dosages were based on those used in the literature and adjusted for body weight (Lin *et al.* 2004). Birds therefore received 0, 1, 1.5, or 2 mg CORT/food bowl/day in these respective dosage subgroups. Because we expected additional within-dosage subgroup variation in CORT concentrations due to between-individual differences in food consumption, we included 4 birds in the low, intermediate, and high dosage subgroups, and included 3 birds in the control subgroup (n = 15 food birds). For most birds the 50 g portion of pigeon food was *ad libitum* (control birds ate on average 17.5 ± SE 0.48 g/day), although towards the end of the experiment, a few CORT-treated birds increased their food intake so dramatically that on a few days their bowls were completely emptied. On average, however, the highest week's average daily food consumption for "food" subgroup females receiving the highest CORT dosage was below the maximum of 50 g (mean 34.3 ± SE 2.59 g/day).

The food treatment birds were housed identically to all other birds (paired in individual cages), except that females were separated from males by a fine mesh partition for most of the day so that males could not access CORT-treated food but still maintain visual, auditory and some tactile contact with their partner. Each morning (before fresh food was placed in the cage), this partition was removed for two hours so that pairs were free to copulate.

(d) Surgery and blood sampling

We implanted pellets and fat implants subcutaneously on the right flank under the wing after numbing the area with lidocaine (Xylocaine®, 10%). We closed incisions with two stitches and wound glue (Liquid Protect, Hansaplast®), and checked wounds daily during the first week.

All blood samples were taken within 3 minutes of entering the room to avoid CORT elevation due to a stress response (Wingfield *et al.* 1982). All birds were blood sampled ten times during the experiment in addition to a baseline blood sample (day 0 sample) taken just before initiating hormone treatment. Initial baseline pre-treatment CORT concentrations did not differ between treatments or dosages (GLM: treatment: t = 1.211, p = 0.235; dosage: t = –0.552, p = 0.585).

The first three samples after CORT treatment began were collected on days 1-3 in all birds. Birds were then split into 3 groups with different sampling schedules each containing one-third of the birds from each room with equal representation of all treatment methods and dosages. The sampling schemes for these groups were staggered by one day so that only one group was sampled on a given day. Samples 4–7 were collected with between-sample intervals of three days; samples 8–10 were collected with between-sample intervals of five days (Figs 4.1–4.3). Each sampling group was further divided into three time-blocks (1330, 1530 or 1730), when the diurnal pattern of endogenous CORT is at its nadir (de Souza *et al.* 2001). This way, on a given day, only two birds had to be sampled from each room every two hours,

ensuring that CORT levels in all birds were at baseline upon entering the room. Individual birds were always sampled at the same time of day in the afternoon. Blood samples were taken with a 25 G needle and heparinised microcapillary tubes. Ca. 200 µl blood was drawn from the brachial vein, and was centrifuged at 10000 rpm for 10 minutes within 30 minutes of collection. Plasma was separated and frozen at -20°C until hormone extraction.

We measured body mass (to the nearest 0.01g) and tarsus (to the nearest 0.01 mm) of birds on the day that treatment began and measured body mass four additional times over the course of the experiment (days 13, 17, 20 and 30).

II-2 Eggs

Eggs were collected every day in the mornings and first eggs were replaced with an artificial egg until the second egg was laid, after which both were removed. Females were allowed to continue egg-laying and all subsequent clutches were removed in the same manner.

II-3 Plasma hormone analysis

Plasma CORT extraction and assay protocols were adapted from the MPI of Ornithology, Andechs Steroid Hormone Assay Protocol described in Goymann *et al.* (2006). In addition to the double dichloromethane extraction, we performed a single methanol extraction in which 1 ml of 70% MetOH was added to extracts, vortexed and placed in –20°C overnight. The following morning, these samples were centrifuged for 5 minutes at 2000 rpm at 4°C, the MetOH phase was decanted and dried under a nitrogen evaporator. Extracted hormone was re-suspended in 300 µl PBS. Recoveries were on average 78.8% ± SE 0.42. The assay was validated for parallelism by measuring serial dilutions of plasma samples containing high concentrations of CORT. Individuals' samples were evenly distributed to different assays with respect to treatment groups and dosages. Average intra-assay coefficient of variation was 18.6% and inter-assay variation was 10.2%.

II-4 Statistical analyses

We used the software R (R Development Core Team 2008) for all statistical models. For mixed effects regressions (LMER function) we used the lme4 package (Bates *et al.* 2008) and languageR (Baayen 2008). All tests were two-tailed with significance delimited by alpha = 0.05.

We quantified maternal plasma CORT concentrations for the full duration of the experiment by integrating the area under each individual's hormone curve ("total CORT exposure"). Individual profiles were constructed using the 11 blood samples from each bird over the course of the experiment. CORT profile graphs were created using SigmaPlot and exported as JPEGs into ImageJ (freeware produced by NIH: http://rsb.info.nih.gov/ij/) with which we estimate as the dependent

variable, with initial body mass and tarsus as covariates in the model (correlation between initial body mass and tarsus: $r = 0.61$).
To determine whether treatment methods yield differences in duration of plasma CORT elevation, we first had to correct duration of CORT elevation for the difference in hormone release rates because hormone release rates varied between treatments (in intermediate dosage: $t = -2.479$, $n = 10$, $p = 0.048$; high dosage: $t = -2.473$, $n = 10$, $p = 0.043$; but not the low dosage: t = –1.464, n = 10, p = 0.180) as well as between dosages (fat implants: $t = 5.54$, $n = 9$, $p < 0.001$; pellets: $t = 6.01$, $n = 12$, $p < 0.001$; food group: $t = 6.14$, $n = 15$, $p < 0.001$). Therefore, instead of using dosage, we used the more direct measure of total CORT exposure (integrated CORT concentrations during the entire experiment) as a measure of CORT exposure experienced by birds, and used this to correct duration of plasma CORT elevation.

We quantified duration of elevation by calculating the 95th percentile CORT concentration based on all control birds for each of the 10 hormone sampling points during the CORT treatment period and used this as a threshold value to delimit significant CORT elevation over baseline concentrations. For each experimental individual, we recorded the day of the latest sampling point for which CORT was still elevated above this threshold value. Because the three treatment methods differed in total CORT exposure (LMER predicting total CORT exposure with treatment method as a fixed factor and dosage as a random factor: $t = -3.45$, $n = 36$, $p = 0.002$), and because total CORT exposure was highly correlated with duration of elevation (Pearson's $r = 0.79$, $n = 36$, $p < 0.05$), we then standardized the three treatment methods with respect to total CORT exposure in order to isolate treatment method-related differences in duration of CORT elevation from the effect of actual hormone release rates since the first is the main focus of our study. We therefore regressed duration of CORT elevation on CORT exposure to extract residual duration of CORT elevation.

Our second main objective was to test the relative importance of duration of CORT elevation vs. absolute CORT exposure on reproductive output and body condition. To separate the highly correlated predictors, we regressed CORT exposure on duration of CORT elevation (because duration of CORT elevation is actually more likely to be the causal factor underlying the correlation between the two factors) and extracted residual CORT exposure for each individual.

III. Results

III-1 Effects of treatment methods on corticosterone levels

To test the effects of our experimental hormone administration on individual CORT profiles (Figs 4.1–4.3) we fitted a linear mixed effects regression (LMER) to the 11 CORT measurements collected from each individual over the course of the

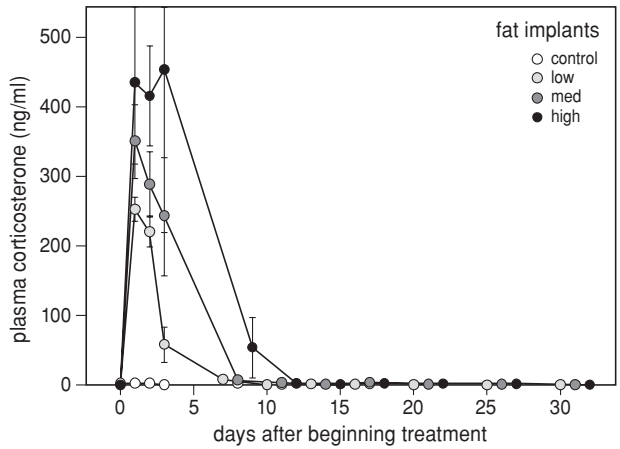

Figure 4.1. Mean (± SE) plasma corticosterone concentrations in fat-implanted birds.

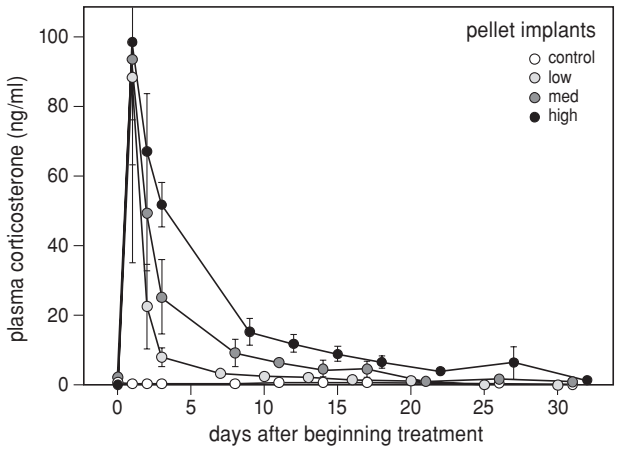

Figure 4.2. Mean (± SE) plasma corticosterone concentrations in pellet-implanted birds.

experiment, including treatment, dosage, sampling day, and their interactions as fixed factors, and individual ID as a random factor. The dosage variable ranked the concentrations of administered hormone for all treatment methods (control = 0, low = 1, intermediate = 2, high = 3). Birds treated with CORT via different treatment methods showed a different relationship between duration of CORT elevation over time and dosage (Figs 4.1-4.3; treatment*dosage*time: $t = 2.022$, $n = 36$ birds, $p = 0.043$).

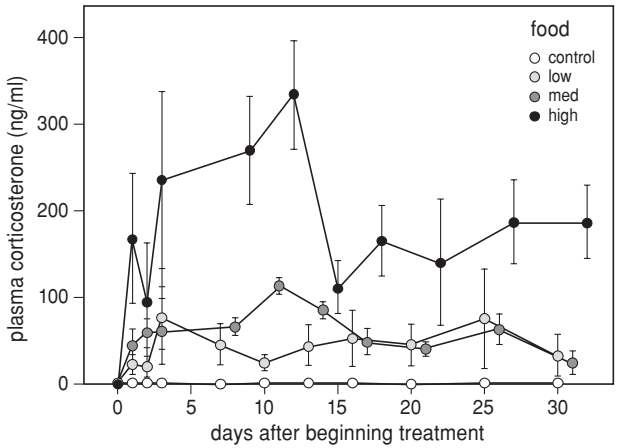

Figure 4.3. Mean (± SE) plasma corticosterone concentrations in birds treated with corticosterone via food.

We were most interested, however, in determining whether treatment methods yield differences in duration of plasma CORT elevation regardless of hormone release rates (in the previous model represented by dosage). We performed a separate regression predicting residual duration of CORT elevation (corrected for total CORT exposure) for all CORT-treated birds, with treatment as a fixed factor. Birds treated with CORT via the food showed longest residual duration of CORT elevation, followed by pellet-implanted birds and butter-implanted birds (GLM: $t = -5.67$, $n = 36$, $p < 0.001$; means ± SE: fat implants 6.17 ± 1.56; pellets 15.67 ± 2.64; food 31.25 ± 0.25; post-hoc tests: fat implants vs. food: t = –10.305, $n = 24$, $p < 0.001$; pellets vs. food: $t = -2.24$, $n = 27$, $p = 0.034$; pellets vs. fat implants: $t = 7.90$, $n = 21$, $p < 0.001$). Plasma CORT remained elevated for the entire duration of the experiment in all birds treated with CORT via the food, but not in a single fat-implanted or pellet-implanted bird.

III-1 Effects of duration of corticosterone exposure vs. total corticosterone exposure on reproduction and body condition

We then tested the relative importance of duration of CORT elevation vs. absolute CORT exposure on reproductive output and body condition. We performed two LMERs to predict the number of clutches and the number of eggs laid during the experiment, including residual CORT exposure and duration of CORT elevation as covariates, and treatment method as a random variable. Both the number of clutches and eggs laid showed a negative association with residual CORT exposure (clutches: $t = -2.31$, $n = 36$, $p = 0.027$; eggs: $t = -2.08$, $n = 36$, $p = 0.045$; Fig. 4.7), and no significant relationship with duration of CORT elevation (clutches: $t = -1.63$, $n = 36$, $p = 0.112$; eggs: $t = -1.774$, $n = 36$, $p = 0.085$).

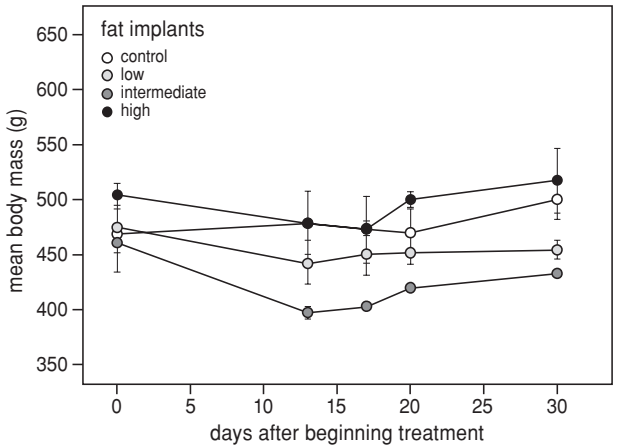

Figure 4.4. Mean (± SE) body mass in fat-implanted birds.

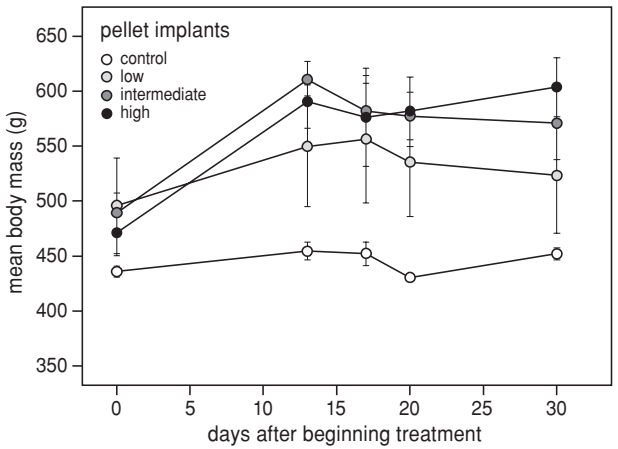

Figure 4.5. Mean (± SE) body mass in pellet-implanted females.

We then performed a LMER to predict change in body condition (Figs 4.4–4.6) over the course of the experiment. We performed the same model as previously for egg-laying, but included integrated total body mass as the outcome variable. In this model, residual CORT exposure was not significant (t = –0.080, n = 36, p = 0.937) but duration of CORT exposure was a strong positive predictor of gain in body condition (t = 3.67, n = 36, p < 0.001). In this model, initial body mass also showed a strong positive relationship with integrated body mass (t = 5.72, n = 36, p < 0.001) but tarsus did not (t = 0.145, n = 36, p = 0.885).

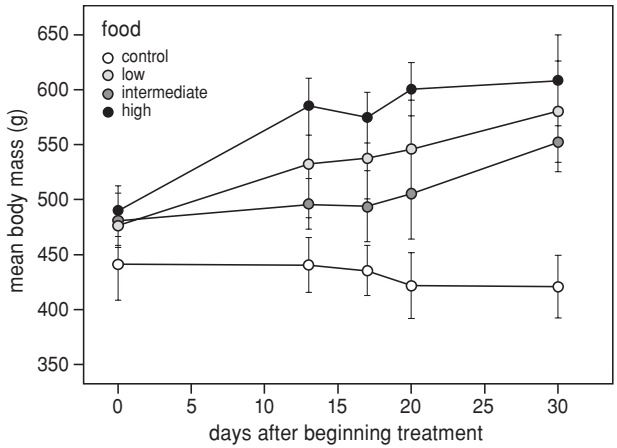

Figure 4.6. Mean (± SE) body mass in birds treated with corticosterone via food.

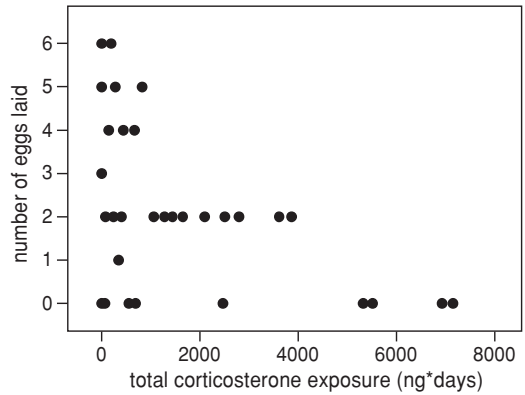

Figure 4.7. Number of eggs laid by each bird as a function of integrated plasma corticosterone concentrations for the entire experiment (32 days).

In the subset of birds treated with CORT via food, we measured daily food intake (Fig. 4.8) and analyzed the relationships between food intake, body condition and CORT in birds from this treatment group. We performed a GLM predicting integrated body mass over the course of the experiment, with average food intake, initial body mass and tarsus as covariates. In this model, average food intake was a very strong predictor of body mass gain over the course of the experiment (GLM: $t = 5.38$, $n = 15$, $p < 0.001$). We also found that average food intake over the course of the experiment was positively associated with total CORT exposure

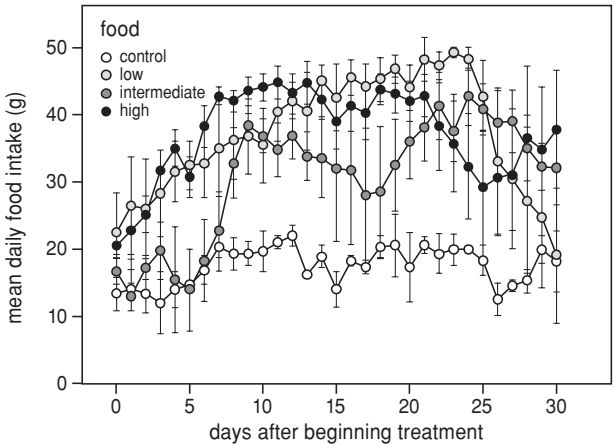

Figure 4.8. Mean (± SE) food intake in birds treated with corticosterone via food.

during the experiment (GLM: $t = 4.83$, $n = 15$, $p < 0.001$; Fig 4.7). Because CORT levels in all birds from this treatment group remained elevated throughout the entire experiment, we did not perform the complementary test of whether food intake was related to residual duration of CORT elevation.

IV. Discussion

IV-1 Effectiveness of the three treatment methods

Successful tests of the regulation and functional consequences of chronic stress rely on the selection of the appropriate experimental CORT treatment method. This method should induce the natural pattern of glucocorticoid exposure characteristic of chronic stress. The CORT profile associated with chronic stress is typically considered to consist of repeated short-term glucorticoid up-regulation, which averages out to fairly consistently elevated CORT exposure over time (e.g. Strochlic & Romero 2008). Our findings clearly indicate, however, that a consistent rate of experimental CORT release is not always reflected as consistently elevated CORT in the blood. At the end of the four-week experiment, both fat implants and pellets were still of substantial size, yet only yielded a ca. one-week peak-like elevation in plasma CORT followed by a rapid return to baseline concentrations. This hormone profile is similar to findings from other studies which implanted birds with the same pellets used in our experiment (Davison *et al.* 1985; Bonier *et al.* 2007; Müller *et al.* 2009; Henriksen *et al.* in prep) as well as studies which implanted birds with osmotic pumps (Etches *et al.* 1984; Davison *et al.* 1985). Clearly, these alternative

implants are no more effective than the Silastic implant (Criscuolo *et al.* 2005; Angelier *et al.* 2007) as all fail to stably elevate CORT over a prolonged period of time, irrespective of induced peak levels.

Interestingly, the birds receiving CORT via food showed consistently high plasma CORT levels throughout the experiment. Other studies which treated birds with CORT via food or water (food: Davison *et al.* 1983; Lin *et al.* 2004; water: Post 2003) show similar success in longer, more stable CORT elevation. After a bird consumes a food item containing CORT, plasma CORT levels increase rapidly and then decrease rapidly back to baseline level usually within the span of one hour (Breuner *et al.* 1998; Schoech *et al.* 2007; Spencer & Verhulst 2007) much like a single acute stress response (Greenberg & Wingfield 1987). Because pigeons eat at least once every hour during daylight (Chabot & Menaker 1992), the birds treated with hormone via the food received frequent small boluses of hormone throughout the day. We suggest that oral CORT administration mimics the natural pulsatile nature of repeated exposure to stressors and that pulsatile CORT delivery is the key to prolonged CORT elevation in the blood. This is supported by the findings from a recent study which treated white-crowned sparrow via repeated daily applications of CORT dissolved in a gel to the skin for two weeks (Busch *et al.* 2008). Each treatment induced a CORT peak at 30 minutes after application which decreased substantially by 45–60 min. This approach was effective in inducing CORT elevation throughout the duration of the experiment. The salient emerging pattern is that CORT delivered in a continuous manner (via implantation) results in a single peak followed by a fast drop back down to baseline levels, and prolonged CORT elevation in the plasma is achieved only when CORT is administered in repeated, discrete pulses. This prompts numerous questions about the mechanisms by which pulsatile CORT treatment bypasses immediate down-regulation of exogenous CORT.

IV-2 Effects of corticosterone exposure vs. duration of corticosterone elevation.

CORT-treated birds from different treatment and dosage groups showed considerable variation in both total CORT exposure (integrated plasma CORT levels over the course of the experiment) as well as in duration of CORT elevation above baseline levels. Although the two variables were correlated, we were able to disentangle them statistically, and demonstrate that they have different physiological consequences. For example, in congruence with other studies (Petitte & Etches 1991; Salvante & Williams 2003) total CORT exposure corrected for duration reduced the number of clutches and the number of eggs laid by females, supporting the hypothesized proximate role of CORT in suppressing reproduction in birds under stress (Wingfield & Sapolsky 2003). Duration of CORT elevation, however, did not significantly predict variation in egg-laying. We also expect that elevated CORT should initiate behavioural and physiological self-maintenance processes that promote a

positive energy balance (Landys *et al.* 2006). This has been supported in previous studies which demonstrate hyperphagia (Koch *et al.* 2002; Holberton *et al.* 2007) and increased fat deposition (Gray *et al.* 1990; Wingfield & Silverin 1986; Berdanier 1989; Rebuffe-Scrive *et al.* 1992) following prolonged exposure to glucorticoids under *ad libitum* food conditions in vertebrates. Interestingly, we found that while duration of CORT elevation positively predicted body mass gain, total CORT exposure controlled for duration did not. Our findings suggest that CORT-mediated modulation of investment in reproduction vs. survival is rooted in different dimensions of the plasma CORT profile: reproduction is regulated mainly by magnitude of CORT elevation while recovery of body condition is driven by temporal distribution of CORT elevation.

IV-3 Corticosterone clearance rates and the role of CBGs

CORT is maintained in circulation by corticosteroid binding globulins (CBGs) which bind to CORT with very high affinity (Breuner & Orchinik 2002). While unbound hormones have a short half-life of about 10 minutes (Kovacs *et al.* 1983; Woodward *et al.* 1991), CBG-bound hormones are effectively protected from degradation in the liver; CBGs therefore modulate how much of the CORT that is released into the blood remains in circulation (Bright & Darmaun 1995). Only the unbound fraction of CORT ("free CORT"), is considered the fraction bio-available to tissues and equilibrates with the bound fraction, depending on the amount of CBG (Mendel 1989). CBG capacity has been shown to shift in response to changes in plasma CORT levels in the direction that would return plasma CORT to the homeostatic range. Suppressing the HPA axis via dexamethasone injection, for example, which halts endogenous CORT production, causes CBG capacity to increase (Adcock *et al.* 2007; Berdusco *et al.* 1995), and ensures the binding of the remaining plasma CORT and therefore maintenance in circulation. CBG capacity decreases, however, in response to acute stressors, which enhances the bio-availability of the hormone (reviewed in Breuner *et al.* 2006) but also would be an effective mechanism to increase the clearance rate of CORT in circulation and reduce total glucocorticoid exposure. Jamieson *et al.* (1999) demonstrated that adrenalectomy reduces bioactivity of 11-HSD-1, an important enzyme for converting glucorticoids into an inactive form. However, this effect was attenuated after dexamethosone replacement, suggesting that hepatic CORT metabolism could increase in response to higher concentrations of free CORT in the blood. We therefore hypothesize that continuous exogenous CORT release at levels detrimental to the organism result in a reduction in CBG capacity, increasing the clearance rate of the hormone. In line with this hypothesis is the finding of Müller *et al.* (2009) who implanted kestrel and owl nestlings with dissolving CORT pellets, and found that CBG capacity was transiently elevated during the CORT peak and returned to a low level concomitant with the drop in plasma CORT levels.

We predict that if plasma CORT levels fluctuate, such as would be the case in birds chronically exposed to frequent stressors, or birds receiving pulsatile CORT treatment, then CBG capacity should be maintained at a higher level than in birds receiving continuous release CORT treatment because of the intermittent return of plasma CORT concentrations to baseline level which should "reset" CBG capacity to normal levels. Indeed, short-term down-regulation of CBG capacity following short-term elevation of CORT does quickly recover after plasma CORT concentrations return to baseline level (Adcock *et al.* 2007). In addition, CORT treatment in a pulsatile manner did not induce a decrease in CBG capacity or an increase in hepatic CORT metabolism (Busch *et al.* 2008).

V. Conclusions

We suggest that pulsatile hormone delivery facilitates the maintenance of higher CBG capacity and that this is the mechanism which retains more exogenous CORT in circulation. While CBG capacity is frequently measured in descriptive field studies, unfortunately it has only rarely been measured in birds receiving experimental CORT treatment although this would provide a simple direct test of this hypothesis. To induce biologically relevant physiological benefits and costs of chronic CORT exposure, experiments should employ appropriate CORT treatment techniques that result in CORT plasma profiles that mimic the consistent long-term elevation of chronically stressed birds. The implantation approach is clearly not useful for experiments testing effects of the prolonged glucocorticoid exposure associated with chronic stress, and we suggest the use of alternative methods which administer CORT in a pulsatile manner such as via food or drinking water, or via repeated application of gel containing dissolved CORT to the skin (Busch *et al.* 2008). The obvious limitation is the difficulty in applying this approach in experiments using free-living birds. Finally, we show that total CORT exposure and duration of CORT elevation play separate roles in the CORT-mediated regulation of investment towards reproduction vs. survival, underscoring the importance that experimental simulations of chronic stress use a hormone treatment approach that truly induces long-term CORT elevation.

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