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Beeswax corticosterone implants produce long-term elevation of plasma corticosterone and influence condition

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ABSTRACT

Glucocorticoids can play a critical role in modulating life-history trade-offs. However, studying the effects of glucocorticoids on life-history often requires experimentally elevating plasma glucocorticoid concentrations for several weeks within normal physiological limits and without repeated handling of the animal. Recently, implants made of beeswax and testosterone (T) were shown to have release dynamics superior to some currently available T implants, and these beeswax implants dissolved, eliminating the need to recapture the animal. We evaluated the utility of beeswax implants containing four different dosages of corticosterone (CORT; the primary glucocorticoid in birds) and their effect on several condition indices in a captive colony of zebra finches (*Taeniopygia guttata*). The three implants with the greatest CORT doses (0.05, 0.1, and 0.5 mg) produced spikes in plasma CORT concentrations 20 h after treatment, but were within the limits that zebra finches may normally experience. The 0.5 mg CORT implant elevated plasma CORT between typical baseline and restraint stress levels reported in other studies of zebra finches for the entire 35 day experiment. Birds in the 0.5 mg implant group were heavier, had greater furcular fat scores, and had lower hematocrit than birds in the control and other CORT implant groups. Beeswax CORT implants are a low cost method of elevating plasma CORT for a prolonged time. Furthermore, because there is no need to remove these implants at the end of a study, this method may be amenable to studies of free-ranging animals.

1. Introduction

In vertebrates, glucocorticoids are pivotal in regulating daily and seasonal responses to changing energetic demands and in mediating the response to unpredictable events (Landys et al., 2006; Wingfield et al., 1998). Circulating levels of glucocorticoids can change rapidly in response to variations in the physical or social environment, and lead to a suite of physiological and behavioral changes that can influence fitness (Crossin et al., 2016). Recent work in birds has indicated that variation in plasma corticosterone (CORT; the main glucocorticoid in birds) concentrations can influence parental care (Crossin et al., 2012), offspring sex ratios (Bonier et al., 2007), offspring development (Butler et al., 2010; Müller et al., 2009b), immune function (Butler et al., 2010; Råberg et al., 1998) and individual fitness (reviewed in Bonier et al., 2009). Thus, there is keen interest in determining the causal role of this hormone in regulating a range of life-history trade-offs (Crossin et al., 2016). Such experimental manipulations require a means to manipulate plasma CORT in the field and laboratory for prolonged durations.

The growing interest in CORT has led to an increasing number of studies experimentally manipulating plasma CORT concentrations in the field and laboratory, but such manipulations are frequently problematic (Crossin et al., 2016; Fusani, 2008). Ideally, a manipulation would elevate plasma CORT consistently within the desired range (between baseline and stress-induced levels) over a specific time scale and be minimally invasive. Injections elevate CORT in the short-term, but require repeated handling and capture of birds, which is logistically difficult and the repeated handling and injections could also affect the hypothalamic-pituitary-adrenal axis and therefore plasma CORT concentrations (Loiseau et al., 2008; Müller et al., 2009a). The passive administration of CORT through food or water is non-invasive but requires that animals are held in captivity, and the CORT dose will vary among individuals depending on the amount of water and food consumed (Breuner et al., 1998; Müller et al., 2009a). Oral treatments also produce a high, but relatively brief (≈1 h), spike in plasma CORT (Spencer and Verhulst, 2007). Silastic implants generally elevate

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plasma CORT to acute stress levels for 1–3 days (but see Ouyang et al., 2013) before levels return to baseline (Newman et al., 2010; Shahbazi et al., 2014; Wingfield and Silverin, 1986). Osmotic pumps are relatively large and elevate plasma CORT for over a week, but they have the downside of producing a spike in plasma CORT 24–48 h following implantation (Horton et al., 2007). Osmotic pumps and silastic implants will remain in the animal long-term if it is not recaptured, which could pose ethical issues. Commercially available CORT pellets (hereafter 'pellets') are advantageous because they are biodegradable and, thus, no part remains in the animal long-term, but they are relatively costly compared to silastic implants (Fusani, 2008; Müller et al., 2009a). Pellets also have the benefit of producing a spike in plasma CORT approximately half that produced by a silastic implant and can elevate plasma CORT concentrations from a few days to several weeks (Bonier et al., 2007; Fusani, 2008; Müller et al., 2009b).

Recently, Quispe and colleagues (2015) developed implants made of beeswax to administer testosterone (T). Compared to silastic implants and pellets, beeswax implants produced a lower spike in plasma T 24 h following implantation and more consistently elevated plasma T for 2 weeks following implantation (Quispe et al., 2015). Beeswax implants provide the additional advantage of dissolving inside the animal (Quispe et al., 2015). We evaluated the use of beeswax implants for long-term elevation of plasma CORT using a captive population of zebra finches (*Taeniopygia guttata*). We prepared implants containing four different CORT dosages as well as blank implants and evaluated plasma CORT levels and several condition indices to determine how CORT dosages affected condition. Based on the results of Quispe et al. (2015), we expected that plasma CORT would remain elevated above baseline for 2 weeks following implantation, with an initial spike in plasma CORT within the range produced by acute stress. Because CORT supports gluconeogenesis primarily through the breakdown of lipids and proteins (Sapolsky et al., 2000), we predicted that individuals receiving implants containing more CORT would decrease fat and muscle scores, and lose body mass compared to controls. We further predicted that hematocrit would increase in individuals receiving more concentrated CORT implants because elevated plasma glucocorticoids increase erythropoiesis in poultry and mice (Olanrewaju et al., 2007, 2006; Voorhees et al., 2013).

2. Methods

2.1. Animals, housing conditions, and experimental design

We kept adult zebra finches in temperature-controlled rooms (25 ± 1 °C) on a 14:10 h light: dark cycle with food (mixed seeds; Kaytee, Chilton, WI, USA) and water *ad libitum*. We used 24 male and 24 female zebra finches and randomly assigned each bird to one of six treatment groups while equally distributing the sexes among treatments ($n = 8$ per group): 0.01 mg CORT implant, 0.05 mg CORT implant, 0.1 mg CORT implant, 0.5 mg CORT implant, 0 mg CORT implant (implant control), and no implant (total control). We selected these dosages based on the ability of commercially available implants of similar concentrations to moderately increase plasma CORT in other passerines (Bonier et al., 2007; Pravosudov, 2003). We divided the birds between 8 cages, with each cage including one bird from each treatment group and a variable sex ratio. Cages were distributed among three separate rooms.

Five to 7 days after assigning the birds to cages and allowing them to acclimate to their social group, we collected a pre-treatment blood sample from each bird to quantify baseline plasma CORT. We aimed to bleed birds at each time point within 3 min of entering the room (Romero and Reed, 2005) and staggered bleeds over three consecutive days to achieve this. On a sample day, all of the birds in one cage from each of the three rooms were bled on average 2:13 ± 0.05 min (range 0:53–5:36 min) after entering the room. We collected blood samples between 8:30 AM and 9:45 AM by puncturing the brachial vein with a 26½ gauge needle and collecting ⩽150 μL of blood from each bird in heparinized capillary tubes. Within an hour of collection, we centrifuged samples at 10,000 rpm for 5 min and stored the separated plasma at −80 °C until hormone assay (see below).

Fourteen days after collecting the pre-treatment samples, we inserted one implant into each bird (total control birds received no implant or incision, but were handled similarly to the other birds). To insert implants subcutaneously on the flank, we first swabbed the surgical site with povidone-iodine and topically applied benzocaine anesthetic, then made an incision in the skin. After inserting the implant, we closed the incision using cyanoacrylate adhesive (surgi-loc 2oc, Meridian Animal Health, Omaha, NE, USA) and returned the bird to its cage. We performed all implant surgeries between 1 PM and 3 PM, staggering the birds over three days. The day of implantation was designated as day 0 and we collected the first blood samples the morning following implantation (day 1; an average of 19.5 h after implantation), and subsequent samples on days 7, 20, and 35 post-implantation as described previously.

2.2. Implant preparation

We made the implants following a modification of the protocol described by Quispe et al. (2015). We autoclaved beeswax (90% by weight; Sigma-Aldrich, St. Louis, MO, USA, cat. # 243221) and hardened peanut oil (10% by weight; Sigma-Aldrich, St. Louis, MO, USA, cat. # 93967) in glass vials and mixed them in a water bath at 67 °C. Once the beeswax/peanut oil mixture was melted, we added crystalline CORT (cat. # 27840, Sigma-Aldrich, St. Louis, MO, USA) dissolved in ethanol, and allowed the solution to mix until the ethanol evaporated. We then poured the beeswax/peanut oil solution into a 3 mL syringe with a Luer-lok tip (Becton Dickinson, Franklin Lakes, NJ, USA). Once the mixture had cooled and partially solidified in the syringe, we extruded it through the tip to produce a 1.75 mm diameter cylinder, which we cut into 10 mm lengths. To create implants with different doses of CORT, we made separate batches of the beeswax/peanut oil mixture and to each we added ethanol with sufficient CORT (calculated according to the average mass of a 10 mm long beeswax cylinder and the amount of CORT dissolved in the ethanol) such that a 10 mm length contained 0.01 mg, 0.05 mg, 0.1 mg, or 0.5 mg of CORT. Control implants were made with ethanol that contained no CORT.

2.3. Post-implantation care

We experienced 8% mortality (4 of 48 birds) and two birds had their implant removed prior to the completion of the study due to issues with the incision site. One bird from each group that received an implant containing CORT died but no birds from the implant control group died. These deaths may be due to some combination of infection following implantation, the stress of the surgery, and the effects of elevated CORT. As a result of these deaths, we treated all of the birds with the antibiotic Baytril (at 200 mg/L in water) beginning one week following implantation and had no further mortality. An additional six birds displayed some sickness behavior or issues with their surgical site that led us to individually dose them with Baytril (15 mg/kg). Deaths following implantation have occurred in other studies (Shahbazi et al., 2014), and we encourage future implant studies to report mortality so that

researchers can better evaluate the costs and benefits of utilizing a particular method.

2.4. Condition indices

We examined differences among CORT treatment groups and controls in several condition indices by measuring body mass, fat stores, pectoral muscle size, and hematocrit following each blood sample. Body mass was measured to the nearest 0.1 g using a digital balance. To estimate fat stores, we visually scored the amount of fat in the furcular region by assigning a score of 0–5 (0 represented no fat, 5 represented bulging deposits, Helms and Drury, 1960). We estimated the size of the pectoral muscles on a scale from 0 to 3 (Bairlein, 1995; Davies et al., 2015). Finally, we quantified hematocrit using a micro-hematocrit capillary tube reader after centrifuging samples as described previously.

2.5. Hormone assay

We quantified total plasma CORT levels following double extraction with dichloromethane (average extraction efficiency 76%) using a direct radioimmunoassay (following Wingfield et al., 1992). The average plasma volume was 39.7 ± 0.7 μL (range 11–50 μL) and we ran samples in singlets to increase the detection probability. Sample CORT concentrations were adjusted for extraction efficiency and original plasma volume. We extracted and ran samples in two assays and distributed samples from different treatments equally among the assays. All of the samples from an individual were randomly distributed in the same assay. We calculated the intra-assay variation as the coefficient of variation among standards within the assay and the inter-assay variation using the standards from both assays. The average intra-assay variation was 14.9% and the inter-assay variation was 22.5%. The average detection limit for the assays was 1.54 ng/mL and 53 of the 223 samples quantified fell below this and were assigned the detection limit for the assay.

2.6. Statistical analyses

For CORT, mass, and hematocrit, we compared treatment groups using general linear mixed models. We checked the residuals from these models for normality and performed transformations where appropriate or selected an appropriate distribution. In all models, we nested CORT treatment within room and cage (included as fixed factors) because samples were not entirely independent of each other. In addition to treatment, we included time since implantation, and the interaction between treatment and time since implantation as fixed factors in the model. We also ran the models including the sex x treatment interaction but found no significant differences between the sexes in their responses to the CORT manipulation (all $P \geqslant 0.648$) and present results from the simpler models. Residual CORT concentrations were not normally distributed and we log transformed the raw data to improve model fit. For CORT concentrations, we included the time it took to obtain the sample in seconds as a covariate. The original fat and muscle score values were multiplied by two to produce a scale that consisted entirely of integers. We then used a generalized linear mixed model with a Poisson distribution and a log link function including the factors listed above to assess the effects of treatment on these endpoints. Because of the experimental issues with 10 birds (see post-implantation care above), we ran all analyses including and excluding them and obtained nearly identical results except in the case of body mass (see results). All analyses were performed in SPSS version 23 and we set $\alpha = 0.05$.

3. Results

3.1. Beeswax implants effect on plasma CORT

Plasma CORT concentrations were significantly influenced by the interaction between treatment and time since implantation ($F_{20,\ 150} = 8.449$, $P < 0.001$) and were positively associated with the time that elapsed between entering the room and obtaining the sample (Supplementary Fig. 1, $F_{1,\ 150} = 17.743$, $P < 0.001$). The three implants with the greatest CORT dosages showed a significant initial spike in plasma CORT above both control groups 20 h following implantation (Fig. 1, all $P \leqslant 0.001$). However, only the 0.5 mg implant maintained plasma CORT concentrations at levels above the control groups (all $P \leqslant 0.023$) and the other treatment groups (Fig. 1, all $P \leqslant 0.003$) for the duration of the study.

3.2. Effects of CORT manipulation on condition indices

Several of the condition indices also differed among treatment groups. Using the full data set, we found significant differences among treatment groups in body mass (Fig. 2, $F_{42,\ 151} = 12.001$, $P < 0.001$), but no interaction between treatment and time (all $P > 0.05$). Pre-treatment body mass did not differ significantly among treatment groups (all $P \geqslant 0.231$) nor were there any significant differences between CORT implanted birds and the control groups 20 h following implantation (all $P \geqslant 0.069$). At 7 and 20 days post implantation, birds in the 0.05 mg implant group were significantly lighter than birds in both control group (Fig. 2A all $P \leqslant 0.048$). Birds in the 0.5 mg implant group were significantly heavier than both control groups (both $P \leqslant 0.010$) 7 day post-implantation but there were no other significant differences between the controls and CORT treated birds (all $P \geqslant 0.165$). When we omitted the individuals that died or received individual doses of antibiotics, we detected a significant interaction between CORT treatment and time since implantation ($F_{20,\ 128} = 1.662$, $P = 0.048$) on body mass. Individuals that received the 0.05 mg implant were significantly lighter than total control birds 20 h after implantation ($P = 0.048$). Birds that received the 0.5 mg implant weighed significantly more than either control group at 7 and 20 days post treatment (all $P \leqslant 0.034$). These were the only instances where the implant groups differed from the control groups at the same time point (Fig. 2A).

Similar to the results for mass, we found significant differences among treatment groups in furcular fat scores (Fig 2B, $\chi 2 = 17.06$, $df = 5$, $P = 0.004$). Pretreatment fat scores in the 0.5 mg implant group were significantly greater than those in the total controls ($P = 0.032$) but not implant controls or any other treatment group (all $P \geqslant 0.139$), and did not differ significantly from the controls 20 h after implantation (both $P \geqslant 0.060$). At 7 days post-implantation, fat scores were significantly greater in the 0.5 mg implant group than those in control or other implanted groups (all $P \leqslant 0.017$). Fat scores in the 0.5 mg implant group remained significantly greater than those from the two control groups at 20 days (all $P \leqslant 0.003$) and this difference remained significant at day 35 compared to the total control group ($P = 0.032$). The fat scores of the other CORT treatment groups did not differ significantly from the two control groups prior to treatment or at any point following treatment (all $P \geqslant 0.107$). We found no effect of the treatment or time since implantation on pectoral muscle scores ($\chi 2 = 1.650$, $df = 1$, $P = 0.895$).

Hematocrit was significantly affected by the interaction between CORT treatment and time since implantation (Fig 3, $F_{20,\ 147} = 4.742$, $P < 0.001$). There were no pretreatment differences among groups in hematocrit (all $P \geqslant 0.115$) nor were there significant differences between the control groups and the CORT

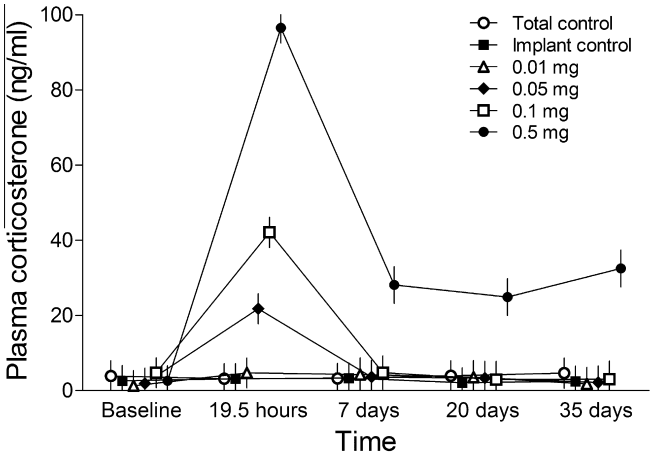

Fig. 1. Plasma corticosterone of zebra finches increased within 20 h of implantation in the three highest CORT treatment groups. The 0.5 mg implant elevated plasma CORT above control and other CORT treatment groups between 7 and 35 days following implantation. Points show least square means and SEM, and are separated along the horizontal axis for visual clarity.

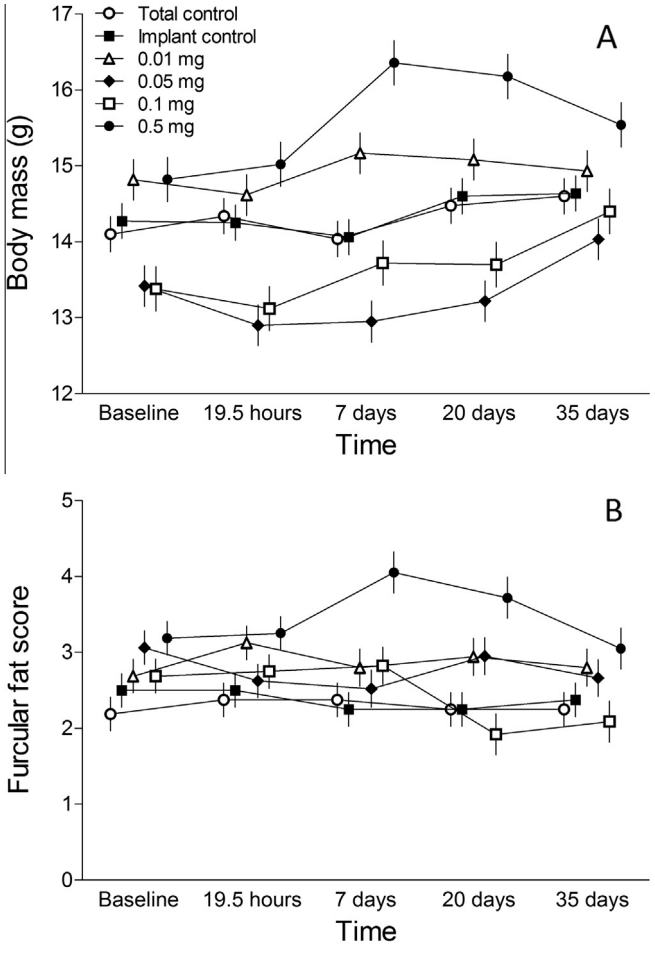

Fig. 2. Corticosterone (CORT) treatment significantly elevated mass (A) and fat (B) in zebra finches receiving 0.5 mg CORT implants compared to other treatment groups. Points show mean and SEM, and are separated along the horizontal axis for visual clarity.

treatment groups 20 h post-implantation (all $P \geqslant 0.121$). In the 0.5 mg group, hematocrit declined significantly from 20 h following implantation to 7 days post-implantation ($P = 0.001$) and was

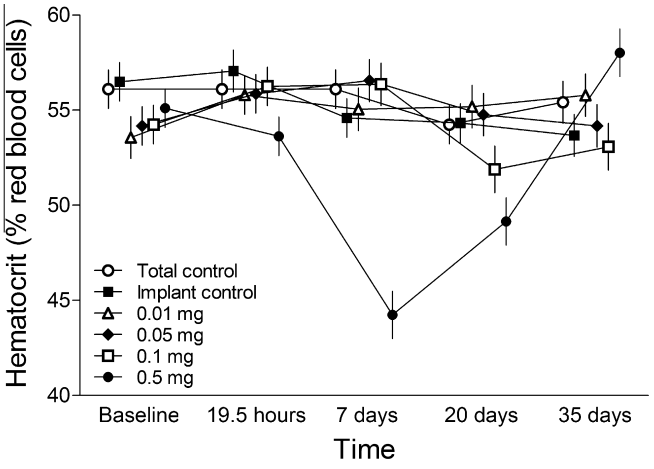

Fig. 3. Corticosterone (CORT) treatment significantly decreased hematocrit in zebra finches receiving 0.5 mg CORT implants 7 and 20 days post-implantation compared to other treatment groups. Points show mean and SEM, and are separated along the horizontal axis for visual clarity.

significantly lower than control and other CORT implanted treatment groups at 7 days (all $P \leqslant 0.001$) and remained significantly lower than controls on day 20 (both $P \leqslant 0.022$). Hematocrit did not differ significantly between the other CORT treatment groups and controls at any sampling point (all $P \geqslant 0.210$).

4. Discussion

4.1. Effects of beeswax CORT implants on plasma CORT concentrations

We evaluated the utility of beeswax CORT implants for long-term, stable elevation of plasma CORT. One potential benefit of the beeswax implants described in Quispe et al. (2015) was a peak in plasma T concentrations 24 h after implantation that was within normal physiological limits, which is in contrast to silastic implants and pellets that produced supra-physiological peaks. Three of the CORT beeswax implants produced spikes in plasma CORT 20 h following implantation. The 0.05 and the 0.1 mg dosages produced peaks in plasma CORT within normal handling restraint induced CORT concentrations which can reach 64 ng/mL in zebra finches (Wada et al., 2008). The initial spike of around 100 ng/mL CORT for the 0.5 mg implant group is within the range zebra finches are capable of producing when injected with adrenocorticotropic hormone (ACTH) but are greater than those produced by handling restraint (Kriengwatana et al., 2014; Wada et al., 2008). Handling restraint for 30 min produced CORT concentrations in adult zebra finches that ranged from 4.97 to 64 ng/mL (mean: 15.52 ng/mL) (Wada et al., 2008), and Kriengwatana et al. (2014) reported that adrenocorticotropic hormone (ACTH) injection elevated plasma CORT an additional 40–50 ng/mL above 30 min stressed induced concentrations. This would place maximum CORT levels around 114 ng/mL in zebra finches, just above the peak in plasma CORT produced by the 0.5 mg implant. This difference between CORT and T implants may be attributed to slight methodological differences between the studies. In contrast to our study, Quispe et al. (2015) soaked the beeswax T implants overnight in saline prior to implantation. It is possible that soaking reduces the initial spike in plasma hormones, but caution should be used because soaking may alter other aspects of CORT release or change the duration that plasma CORT remains elevated. The difference in the size of the peaks may also be attributable to differences in the way CORT and T are metabolized and regulated by birds (Klusonova et al., 2008; Soma et al., 1999).

For three of the beeswax implant groups, plasma CORT returned to baseline levels within 7 days, but the 0.5 mg implant maintained CORT concentrations between 24 and 32 ng/mL for 35 days. These concentrations are between the baseline range of 25–30 ng/mL reported for zebra finches by Shahbazi et al. (2014) and the stress-induced range of up to 64 ng/mL reported by Wada et al. (2008). By day 35, implants were completely dissolved in 24 birds and reduced in size in the remaining 12 implanted birds. Thus, beeswax implants provide a potential method to elevate plasma CORT levels over weeks rather than days, with limited handling of the bird. It is possible that additional modifications of the dose of CORT in the implant or the shape of the implant will allow more modest, long-term elevations in plasma CORT.

4.2. Effects of CORT treatment on condition indices

Glucocorticoids promote fat and protein catabolism to produce glucose (Altuna et al., 2006; Sapolsky et al., 2000). Thus, we predicted birds would lose fat, pectoral muscle, and body mass due to protein and lipid catabolism. However birds in the 0.5 mg CORT treatment group increased furcular fat scores and body mass compared to other groups, and there was no effect of CORT treatment on muscle scores. In birds, CORT can stimulate foraging (Bonier et al., 2011; Crossin et al., 2012; Landys et al., 2004; Pravosudov, 2003), and white-crowned sparrows (*Zonotrichia leucophrys*) and mountain chickadees (*Poecile gambeli*) given CORT implants had higher fat scores than control birds (Landys et al., 2004; Pravosudov, 2003). In our captive study, where birds were provided *ad libitum* access to food, a CORT-induced increase in foraging could explain the body mass and fat gains we detected. This study could be repeated without *ad libitum* access to food to determine if these CORT implants produce the predicted decreases in fat, muscle, and body mass.

Birds receiving the 0.5 mg CORT implant showed significant decreases in hematocrit from day 7–20 compared to other treatment groups before returning to a level slightly above the other groups on day 35. A reduction in hematocrit could be due to decreased erythropoiesis or changes in osmoregulation (reviewed in Fair et al., 2007). However, domestic chickens that were implanted with osmotic pumps containing ACTH for 7 days modestly increased hematocrit by ≈4%, which was attributed to increased erythropoiesis (Olanrewaju et al., 2007, 2006). Chickens that received ACTH via osmotic pumps also exhibited polydipsia (increased thirst) and polyuria (increased uric acid production, Puvadolpirod and Thaxton, 2000), and it is possible that the zebra finches in our study had polydipsia but were limited in their ability to eliminate excess water due to adaptations for water conservation associated with their native, arid habitat. Osmoregulation could also be affected by CORT binding to either mineralocorticoid receptors (MR) or glucocorticoid receptors (GR) in the kidney. House sparrow (*Passer domesticus*) kidneys contain both receptor types (Lattin et al., 2012) and in domestic ducks (*Anas playtrhynchos*), baseline CORT binds to the MR and promotes Na+ excretion and water loss (Thomas and Phillips, 1975a,b). It is possible that binding to the GR produces different effects on osmoregulation (Lattin et al., 2012), and the GR would likely have been bound in the 0.5 mg implant group. Relatively few studies have experimentally manipulated plasma CORT and assessed changes in hematocrit, erythropoiesis, or osmoregulation in songbirds, and this topic warrants further study.

4.3. Conclusion

Beeswax implants provide a means to elevate plasma T reliably for 2 weeks (Quispe et al., 2015) and plasma CORT (this study) for 35 days. These implants provide additional benefits in that they dissolve which makes their use minimally invasive. Three of the beeswax CORT implants produced a spike in plasma CORT 20 h following implantation. Two of these peaks were within handling restraint induced CORT concentrations (Wada et al., 2008), and the third peak was just below CORT levels found in zebra finches following an ACTH injection (Kriengwatana et al., 2014). Prior to use in other species, we recommend initially testing the beeswax CORT implants in captive birds, and monitoring the implanted individuals' plasma CORT, behavior, and condition. This should ensure that CORT is manipulated within the desired range of basal, handling restraint induced, or chronic stress levels depending on the research focus and species. Beeswax implants containing steroid hormones may be a powerful, low cost tool for examining physiological mechanisms underlying life-history trade-offs in birds and other vertebrates.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.ygcen.2016.05.021.

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