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RESEARCH ARTICLE

Physiological trade-offs in self-maintenance: plumage molt and stress physiology in birds

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SUMMARY

Trade-offs between self-maintenance processes can affect life-history evolution. Integument replacement and the stress response both promote self-maintenance and affect survival in vertebrates. Relationships between the two processes have been studied most extensively in birds, where hormonal stress suppression is down regulated during molt in seasonal species, suggesting a resource-based trade-off between the two processes. The only species found to differ are the rock dove and Eurasian tree sparrow, at least one of which performs a very slow molt that may reduce resource demands during feather growth, permitting investment in the stress response. To test for the presence of a molt–stress response trade-off, we measured hormonal stress responsiveness during and outside molt in two additional species with extended molts, red crossbills (*Loxia curvirostra*) and zebra finches (*Taeniopygia guttata*). We found that both species maintain hormonal stress responsiveness during molt. Further, a comparative analysis of all available species revealed a strong relationship between molt duration and degree of hormonal suppression. Though our results support trade-off hypotheses, these data can also be explained by alternative hypotheses that have not been formally addressed in the literature. We found a strong relationship between stress suppression and seasonality of breeding and evidence suggesting that the degree of suppression may be either locally adaptable or plastic and responsive to local environmental conditions. We hypothesize that environmental unpredictability favors extended molt duration, which in turn allows for maintenance of the hormonal stress response, and discuss implications of a possible trade-off for the evolution of molt schedules.

Supplementary material available online at <http://jeb.biologists.org/cgi/content/full/214/16/2768/DC1>

Key words: corticosterone, corticosteroid-binding globulin, environmental predictability, opportunism, protein, red crossbill, zebra finch.

INTRODUCTION

Functional interactions between life-history traits (e.g. trade-offs) can drive evolution, and are a key aspect of evolutionary mechanisms in general (Roff, 1992; Stearns, 1989; Zera and Harshman, 2001). Most trade-offs discussed in the literature focus on investment in reproductive effort (e.g. r and K selection theory) or current vs future reproductive effort (Zera and Harshman, 2001). Trade-offs between different survival-enhancing activities are also important to life-history evolution in long-lived organisms, but they have received comparatively less attention. Two survival-enhancing processes potentially constrained by a trade-off are integument growth/maintenance and the physiological response to stressors: protein availability is suspected to limit investment in these two critical processes (Astheimer et al., 1995).

Maintenance of the integument (e.g. skin, scales, plumage, pelage) is critical for survival and reproduction in animals. Integument quality contributes directly to thermoregulation, predator avoidance and reproduction (Ling, 1970; Stettenheim, 1976). It is particularly important in birds, because flight and insulation depend on plumage quality (Robinson et al., 1976). Plumage molt is expensive: molecular and energetic resources are in high demand and birds often exhibit quiet behaviors that minimize energy use and risk of damage to growing feathers (Hoye and Buttemer, 2011;

King, 1978; Murphy, 1996). Birds forced to molt quickly or with limited food produce lower quality feathers (Dawson, 2004; Murphy et al., 1988) and may have reduced fitness (Hinsley et al., 2003; Nilsson and Svensson, 1996).

Whereas plumage molt represents a relatively long-term investment in survival, the stress response constitutes a suite of physiological and behavioral processes that direct resources towards immediate survival of acute challenges. The hypothalamic–pituitary–adrenal (HPA) axis orchestrates the release of glucocorticoids and corticosteroid-binding globulins (CBG), which support behavioral and physiological adjustments to environmental challenges in all vertebrates (Landys et al., 2006; Wingfield, 2005). Corticosterone (CORT), the primary glucocorticoid in birds, promotes energy release to meet these challenges through several metabolic pathways (Brown et al., 1992; Davenport et al., 1989; De La Cruz et al., 1981; Exton, 1972), with the potential to divert resources from feather growth. CORT also promotes active behaviors (Astheimer et al., 1992; Breuner et al., 1998) that may be incompatible with molt (Murphy, 1996). Large interspecies, inter-individual and seasonal variations exist in both baseline and stress-induced blood levels of CORT and its binding proteins (Landys et al., 2006; Romero, 2002; Wingfield, 2005), but one prevalent pattern is suppression of both baseline and induced CORT levels during

feather molt in birds. Eleven of 13 bird species studied to date show suppressed CORT levels during molt relative to breeding. A resident songbird of the Tibetan plateau (Eurasian tree sparrow, *Passer montanus*) and the non-passerine rock dove (*Columba livia*), however, both maintain baseline and induced levels of CORT during molt (Li et al., 2008; Romero and Wingfield, 2001). Although these may simply be phylogenetic differences, rock doves exhibit a longer, less intense molt than most of the passerine species studied to date; molt details are unavailable for the Tibetan population of tree sparrows. The slower, less resource-intensive feather growth in rock doves may allow maintenance of the hormonal stress response, consistent with a protein-based trade-off (Romero and Wingfield, 2001). Subsequent experiments support this molecular trade-off hypothesis (DesRochers et al., 2009; Romero et al., 2005; Strohlic and Romero, 2008), though plausible alternatives such as energy- or behavior-based trade-offs and more direct mechanistic hypotheses have not been formally considered.

Behavioral, energetic or resource-based conflicts between maintenance processes such as molt and the stress response may have influenced the evolution of life cycle schedules. Species dealing with high exposure to environmental challenges may, for example, have been forced to minimize the rate of resource allocation to feather growth in order to maintain full hormonal stress responsiveness. Red crossbills (*Loxia curvirostra*) and zebra finches (*Taeniopygia guttata*) are two passerine species that exhibit protracted molts. They are both temporally opportunistic breeders that experience relatively high environmental unpredictability. Thus, if a trade-off is mediating investment in molt and hormonal stress responsiveness, we predict that both species will show little suppression of CORT secretion during feather molt. However, zebra finches in different parts of Australia encounter different degrees of seasonality and resource predictability. If stress suppression is either locally adaptable or relatively flexible, then we expect zebra finch populations in more predictable habitats to show greater stress response suppression and/or faster molts. To test these predictions we measured baseline and induced levels of CORT during and outside molt in free-living red crossbills and zebra finches. We also measured CBG to determine whether binding protein capacity and thereby unbound, or free, CORT concentrations change during molt. Finally, to determine whether a relationship exists between molt duration and CORT secretion we then calculated phylogenetically independent contrasts of the 13 species for which data are now available. We also formalize several hypotheses for the resulting patterns, including energetic, molecular and behavioral trade-offs, and discuss these results in the context of these hypotheses as well as possible non-trade-off alternatives.

MATERIALS AND METHODS

Study animals and study sites

Red crossbills (*Loxia curvirostra* Linnaeus 1758) are temporally opportunistic breeders and irruptive nomads that specialize on conifer seeds, which are distributed unpredictably in space and time (Benkman, 1987; Fowells, 1968; Koenig and Knops, 2000; Newton, 1972). Crossbills breed across at least 10 months of the year and may need to move long distances in any season (Adkisson, 1996; Benkman, 1987). A single seasonal pre-basic molt (i.e. post-nuptial molt), in which flight feathers and much of the body plumage are replaced, may be arrested, or paused, for summer breeding (Adkisson, 1996; Hahn, 1995) [for terminology, see Humphrey and Parkes (Humphrey and Parkes, 1956)]. For the present study, data were collected in all months from free-living red crossbills in

Washington, Oregon, California and Wyoming (between 38°N and 49°N latitudes) in 2003–2007.

There are at least 10 vocal ‘types’ of North American red crossbills, in four general size classes (Benkman, 1993; Groth, 1988; Groth, 1993). Variation in body size and bill morphology correlate with presumed adaptation to different conifer species (Benkman, 2003; Groth, 1988; Groth, 1993; Irwin, 2010). In this study we captured 404 adults [26 type 2: large, ponderosa pine-associated birds; 30 type 5: large, lodgepole pine-associated birds; 310 type 3: very small, hemlock/spruce-associated birds; 25 type 4: medium sized Douglas fir-associated birds; and 13 unidentified (see Benkman, 1993; Groth, 1993; Kelsey, 2008)].

Zebra finches (*Taeniopygia guttata*, Vieillot 1817) are opportunistic nomads that feed mostly on grass seeds in both the arid interior and the more temperate, semiarid coastal regions of Australia (Zann, 1996). They can breed in any month and often move long distances to locate recent, rain-induced growth of grasses (Hahn et al., 1997; Zann et al., 1995). Pre-basic molt is aseasonal and takes approximately 10 months to complete (Zann, 1996). Data were collected from free-living adult zebra finches at a relatively predictable habitat (Numurkah, Victoria: 170 km north of Melbourne, 36°08’S, 145°21’E) and a relatively unpredictable habitat in the arid interior (Alice Springs: 66 km southwest of Alice Springs, 23°52’S, 133°28’E) (Perfito et al., 2007). Fifty-four birds were captured at Victoria in winter (July) and spring (October/November) in 2003 and 2004; 17 birds were captured at Alice Springs in spring (October/November) in 2004. Sampling was limited to 4 months of the year, precluding conclusions concerning seasonality of molt and molt duration, but molt in these populations has already been well described across the entire annual schedule. There are no seasonal differences in molt intensity or molt progress, nor is there synchrony of molt across individuals (Zann, 1985; Zann, 1996). Further, no difference has been found in molt duration between the Alice Springs and Victoria populations (Zann, 1996). We therefore consider our limited sample to be an accurate representation of molt across the entire year.

Zebra finch data were collected under the Victorian Department of Sustainability and Environment Wildlife Permit no. 10003656 and the Northern Territory Parks and Wildlife Permit no. 18356. Red crossbill data were collected under UC Davis Animal Care and Use Protocol no. 15059 and all relevant state and federal permits.

Sampling methods

Red crossbills were lured into mist nets using live decoys. Zebra finches were captured in walk-in-traps (Victoria) or mist nets at watering holes (Alice Springs). The alar vein was punctured with a 26 gauge sterile needle and blood was drawn into heparinized microhematocrit tubes (Wingfield and Farner, 1976). Baseline blood samples were obtained within 3 min of capture in a mist net or approach to a seed trap. Although zebra finches captured in seed traps continue foraging and appear calm, the duration from entrance into the trap and investigator approach may allow baseline CORT levels to increase. Traps were observed continuously from a nearby vehicle and time from trap closure to investigator approach varied from 0 to 60 min (median=20 min). There was no significant relationship between the amount of time spent in the trap and baseline CORT levels ($F_{1,51}=0.05$, $r^2=0.003$, $P=0.717$; supplementary material Fig. S1). Birds measured for stress responsiveness were placed in a cotton bag and additional blood samples were collected 10, 30 and 60 min after capture of red crossbills, and 30 min after capture of zebra finches. A total maximum of 150 µl whole blood was collected from zebra finches and 500 µl whole blood from red crossbills. Samples were kept on ice until plasma was separated. Tubes were sealed with

modeling clay and spun 10 min in an IEC clinical centrifuge with a hematocrit head. Plasma was stored at -20°C .

We measured molt intensity as the number of primary flight feathers actively growing (e.g. feather dropped, in pin, or still sheathed) and molt status indicates whether a bird is actively molting primary feathers. Feather molt, however, is a relatively complex process that may interact with stress physiology differently as it progresses. We therefore include molt progress in our analysis to detect potential dynamic interactions. Molt of the nine primary flight feathers proceeds sequentially from wrist to wingtip, and the body plumage, rectrices and other wing feathers sequentially become involved as the primary molt proceeds. Thus, progress through primary molt can provide an estimate of overall molt stage. We determined the position along the wing of the most distally growing feather and then estimated the percentage completion of the growth of this feather. Each feather proximal to this position was scored as 1 (i.e. feather has finished or nearly finished growth) and together they were summed with the progress of the most distally growing feather. Molt progress was therefore scored on a scale from 0.0 (not yet begun) to 9.0 (last primary feather finished growing) (Hahn, 1995). A molt progress of 3.6 means that the fourth most distal primary from the wrist is approximately 60% grown and the three feathers more proximal to it have preceded it in molt sequence. This score would roughly correspond to a 30% completion of primary feather molt and a score of 27 on Newton's scale (Newton and Rothery, 2005).

CORT assay

An enzyme immunoassay kit from Assay Designs (cat. no. 901-097; Ann Arbor, MI, USA) was used to measure CORT in red crossbills as described previously (Cornelius et al., 2010). Plasma dilution and steroid displacement buffer (SDB) concentrations were optimized for red crossbills at 1:40 dilution and 1% (per raw plasma volume) SDB. Samples were randomized and run in duplicate on 32 plates, each with a separate standard curve and hormone standard. Inter-plate variation was 12%, intra-assay variation was 7.3% and detectability was 1.9 ng ml^{-1} and ranged from 0.6 to 4 ng ml^{-1} . Detectability was determined separately for each plate as two standard deviations from the mean of blank wells. Using data directly from the curves to calculate sensitivity is a more conservative and plate-specific method than that provided by the kit manufacturer. Values that were below the limit of detection for each plate were assigned the minimum sensitivity of that plate.

A radioimmunoassay protocol was used to measure CORT in zebra finches as described elsewhere (Wingfield et al., 1992; Tarlow et al., 2003). Samples were measured in two assays and inter-assay variation was 5%, the mean lower detection limit was 1.38 ng ml^{-1} and the mean recovery value was 82%.

CBG assay and free CORT

CBG is a binding protein for steroid hormones (i.e. CORT) in the blood and can fluctuate in binding capacity. The physiological role of CBG and the biological significance of its fluctuations are not entirely understood, but it may regulate the amount of hormone available to tissues, and as such have important implications for behavioral ecology (Breuner and Orchinik, 2002). In crossbills, CBG capacity is known to change with handling time; thus, we sampled CBG capacity at both baseline and 30 min (Breuner et al., 2006). CBG capacity was measured using a tritiated CORT ligand-binding assay as described elsewhere (Breuner and Orchinik, 2001; Lynn et al., 2003). Pooled plasma from free-living red crossbills was used to optimize specific binding at 2 h incubation, 4°C temperature and

1:900 plasma dilution. All samples were run in triplicate and free radioligand was separated from bound by rapid vacuum filtration (Brandel Harvester, www.brandel.com) over glass fiber filters (Whatman GF/B) soaked in 25 nmol l^{-1} Tris and 0.3% PEI for 1 h prior to filtration. Radioactivity was measured using standard liquid scintillation spectroscopy. Specific binding was determined using pooled plasma incubated with 0.9 to 12 nmol l^{-1} [^3H]CORT. The affinity (K_d) estimate for red crossbills was $2.27\pm 0.17\text{ nmol l}^{-1}$. Individual samples were incubated with 20.4 nmol l^{-1} [^3H]CORT, which should occupy approximately 90% of the total binding sites (B_{max}). Thus, capacity values were adjusted to 100% for free CORT calculations. All point samples were run in a single assay on 33 filters. Inter-filter variation was determined using plasma standards and was 14%. Within triplicate variation was less than 7%. Free CORT concentration was estimated using the equation of Barsano and Baumann (Barsano and Baumann, 1989) as in Lynn et al. (Lynn et al., 2003).

Molt–CORT comparison across species

We used published molt durations and stress data during and preceding molt to characterize the molt–CORT relationship for as many bird species as possible (Table 1). Comparisons of hormone data from different studies must be interpreted with caution given that many factors can vary between studies. All 13 studies included here used a standard handling stress protocol (Wingfield, 1983) with baseline CORT samples collected within 3 min of capture, but different sampling times were used to detect maximum CORT. We chose the 30 min sample as a standardized measure for maximum CORT because all 13 studies include a 30 min sample. Sampling date also varied between studies. Here we focused on the breeding–molt transition given the overwhelming bias towards this phase in seasonal stress studies. Non-molt data were taken from active breeders or late breeders that were not yet molting. Most studies did not further sub-divide breeding into specific phases, but if they did (e.g. early and late breeding) the values for those stages were averaged. Very few studies indicated a specific stage of molt; thus, molt was defined only as active growth of flight feathers. All data were collected in the field except in European starlings, which were collected from captive birds. Exclusion of these captive data does not change the results. To standardize for species and intra-assay variation in absolute concentrations of plasma CORT, we report the percentage suppression for each species. Percentage suppression was calculated using the following equation:

$$\% \text{Suppression} = \left(1 - \frac{\text{CORT during molt}}{\text{CORT pre-molt}} \right) \times 100. \quad (1)$$

Data from Victoria and Alice Springs zebra finches were combined for this analysis given that sample sizes allow only preliminary population-level analyses. Percentage suppression was calculated separately for baseline and maximum CORT. Values are given in Table 1.

Statistics

Data were tested for normality using the Shapiro–Wilks test and all hormone levels and CBG capacities were square-root transformed to improve distributions for parametric analyses. We measured baseline and induced CORT levels in each individual, so we used planned pairwise comparisons in a repeated-measures ANOVA to test whether molt intensity or molt progress affects baseline CORT, induced CORT, or the pattern of change in CORT during handling stress. Time since capture was the within-subjects variable and molt intensity and molt progress were between-subjects variables. Tests

Table 1. Species comparison of molt duration and degree of stress suppression

Species	Molt duration (months)	Molt references	%Suppression baseline CORT	%Suppression maximum CORT	CORT references
Red crossbill (<i>L. curvirostra</i>)	5.0	This study	15	-15	This study
Zebra finch (<i>T. guttata</i>)	8.0	Zann, 1985	6	-9	This study
Rock dove (<i>C. livia</i>)	7.0	Johnston and Janiga, 1995	0	-20	Romero and Wingfield, 2001
Gambel's white crowned sparrows (<i>Z. leucophrys</i>)	1.2	Wingfield and Farner, 1979	75	94	Astheimer et al., 1994
European starling (<i>S. vulgaris</i>)	3.3	Dawson, 1994	42	38	Romero and Ramage-Healey, 2000
Redpoll (<i>A. flammea</i>)	1.6	Knox and Lowther, 2000	29	77	Romero et al., 1998
Lapland longspur (<i>C. lapponicus</i>)	1.7	Williamson and Emison, 1971	47	38	Astheimer et al., 1995
Snow bunting (<i>P. nivalis</i>)	1.0	Lyon and Montgomerie, 1995	71	70	Romero et al., 1998
House sparrow (<i>P. domesticus</i>)	2.8	Lowther and Cink, 1992	50	40	Romero, 2006
Grey crowned rosy finch (<i>L. tephroticus</i>)	2.0	Macdougall-Shakleton and Hahn, 2000	-13	34	Richardson, 2003
Eurasian tree sparrow (<i>P. montanus</i>)	2.5	Barlow and Leckie, 2000	-14	0	Li et al., 2008
Rufous collared sparrow (<i>Z. capensis</i>)	1.7	Miller, 1961	81	62	Wada et al., 2006
Cassin's finch (<i>C. cassinii</i>)	2.3	Hahn, 1996	5	47	Richardson, 2003

CORT, corticosterone.

were followed by Holm–Bonferroni correction for multiple comparisons. Significant relationships were further analyzed using individual ANOVA and *post hoc* Tukey comparisons of means. We used JMP v5.0 for statistical comparisons.

The relationship between molt duration and percentage stress suppression across species was assessed using linear regression analysis. We assessed phylogenetic contributions to the molt–CORT relationship by first calculating phylogenetically independent contrasts (PIC) for molt duration and percentage hormonal stress suppression using Mesquite and the PDTree program (Maddison and Maddison, 2006; Midford et al., 2005) and then performing a regression on the contrasts for both baseline and induced CORT. The phylogeny used in the analysis was adapted from Sibley and Ahlquist and is based on DNA–DNA hybridization (Fig. 4) (Sibley and Ahlquist, 1990).

Reliable divergence estimates were not available for many relationships on this phylogeny; thus, we set all branch lengths equal to one. Previous studies suggest that actual branch lengths have little effect on the main outcome of these phylogenetic analyses (Diaz-Uriarte and Garland, 1998; Martins and Garland, 1991).

RESULTS

Red crossbill molt

Similar to previous findings, month predicts primary molt progress ($F_{11,397}=181$, $r^2=0.84$, $P<0.0001$; Fig. 1A). Molt duration (June to November, with a few individuals molting in December) was approximately 5 months across the populations measured in this study. Molt intensity was also predicted by month ($F_{11,400}=43.4$, $r^2=0.55$, $P<0.0001$; Fig. 1B). Mean molt intensity significantly varied

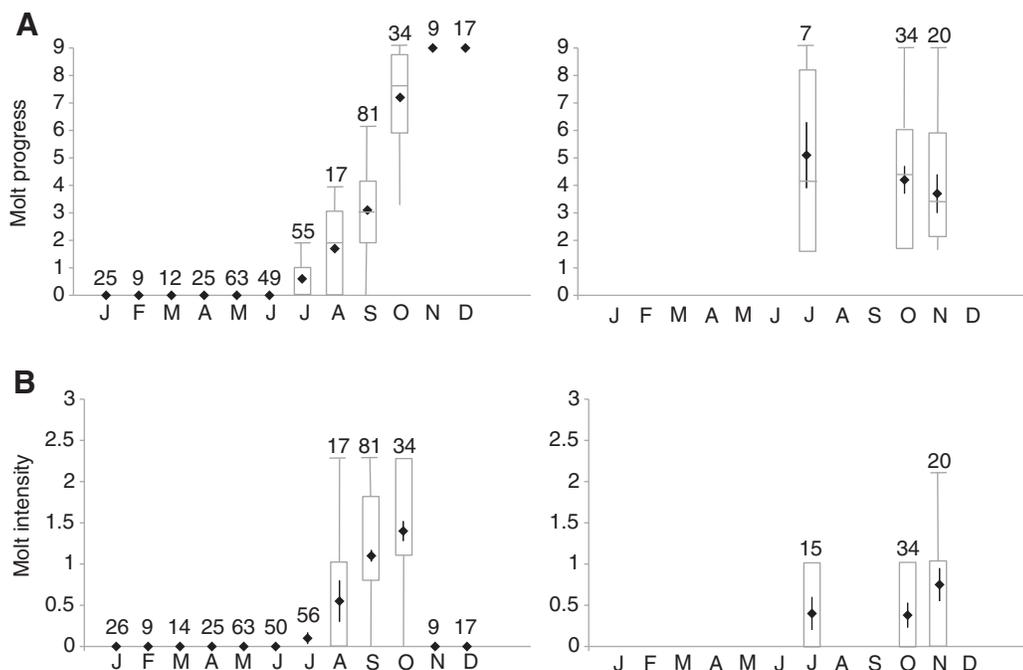


Fig. 1. Primary molt in red crossbills (left) and zebra finches (right). Progress through primary molt (A) shows a seasonal molt in red crossbills and an aseasonal molt in zebra finches. Molt intensity (B) demonstrates that red crossbills have slightly higher molt intensities than do zebra finches. Box and whisker plots, means and s.e.m. bars are given for molt progress and intensity (A and B). Sample sizes are given for all months.

from 0.0 (January to May) to a peak of 1.30 in October. Of 110 individuals displaying active molt, 73 (66%) had one primary feather growing, 36 (33%) had two primary feathers growing, and one (1%) had three primary feathers growing.

Zebra finch molt

In our study molt did not vary across sampling months ($F_{2,60}=0.66$, $r^2=0.02$, $P=0.52$; Fig. 1A). The number of primaries growing on average (molt intensity) in November (0.6) was not significantly different from that in October (0.35) or July (0.4; Fig. 1B), though sample sizes are small. Of 32 actively molting individuals, 31 (97%) were replacing a single primary feather and one (3%) was replacing two primaries simultaneously. Molt duration could not be estimated in this study because sampling did not occur year round and individuals were not recaptured. A molt duration of 10 months is therefore taken from previous research on these populations (Zann, 1985; Zann, 1996).

Total CORT

In red crossbills, total plasma CORT concentration increased with handling time (Fig. 2B; $F_{1,163}=942$, $P<0.0001$). Molt intensity and molt progress had no effect on total baseline or induced CORT levels between subjects (Fig. 2B; $F_{2,163}=0.13$, $P=0.87$ and $F_{1,163}=0.21$, $P=0.65$, respectively) and had no effect on the increase in CORT during handling stress within subjects ($F_{2,163}=0.69$, $P=0.49$ and $F_{1,163}=0.07$, $P=0.79$, respectively).

Zebra finches in this study (except for a single individual that had two feathers growing) replaced only a single feather at a time. We therefore used molt status (i.e. molting or not molting) rather than molt intensity in the repeated measures ANOVA. Total plasma CORT concentration increased with handling time (Fig. 2A; $F_{1,53}=60.5$, $P<0.0001$). Molt status and molt progress had no effect on total baseline or induced CORT levels between subjects (Fig. 2A; $F_{1,53}=0.64$, $P=0.43$ and $F_{1,53}=0.02$, $P=0.88$, respectively) and had no effect on the increase in CORT during handling within subjects ($F_{1,53}=1.16$, $P=0.29$ and $F_{1,53}=3.2$, $P=0.08$, respectively).

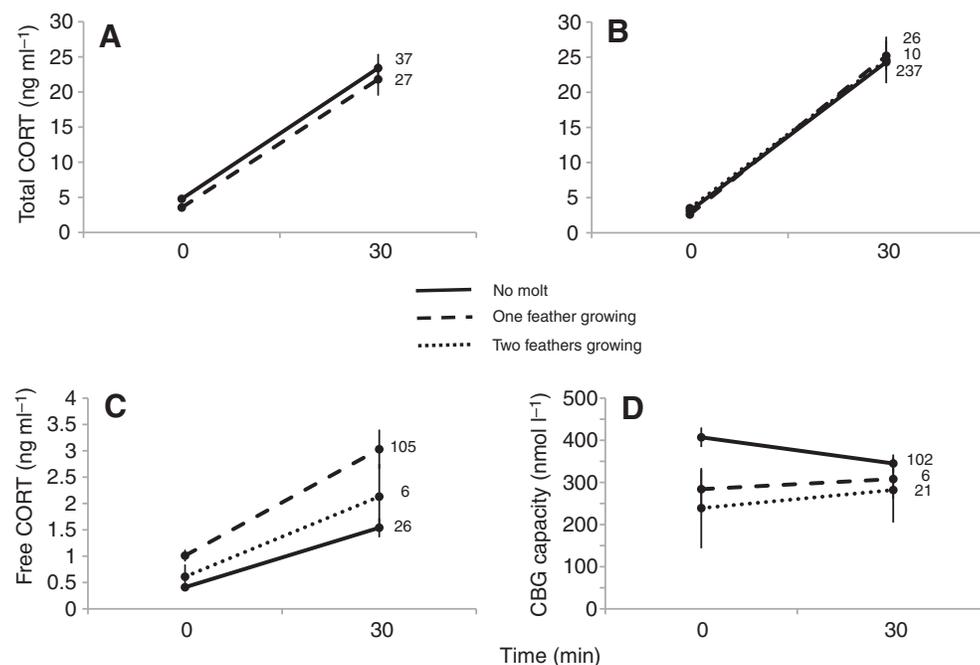


Fig. 2. Changes in corticosterone (CORT) physiology during handling stress in zebra finches and red crossbills across different molt intensities. CORT increases during handling stress (A, zebra finch; B, red crossbill; C, red crossbill free CORT; $P<0.0001$) and corticosteroid binding globulin (CBG) tends to decline (D, red crossbill CBG capacity; $P=0.03$; Holm–Bonferroni $\alpha<0.025$). Total CORT does not vary by molt intensity (i.e. if birds are not molting or have either one or two primary flight feathers growing simultaneously) in zebra finches (A) or red crossbills (B). CBG capacity, however, declines with increasing molt intensity (D; $P=0.02$) and free CORT increases with molt intensity in red crossbills (C; $P=0.002$). Sample sizes are given for each condition.

CBG and free CORT

CBG capacity and free CORT were not measured for zebra finches in this study. In red crossbills, free CORT plasma concentration (i.e. CORT that is not bound to CBG) increased during handling (Fig. 2C; $F_{1,123}=28.7$, $P<0.0001$) and there was a strong trend for CBG capacity to decrease during handling (Fig. 2D; $F_{1,97}=5.02$, $P=0.03$, Holm–Bonferroni $\alpha<0.025$). CBG capacity was lower in molting individuals (Fig. 2D; $F_{2,97}=3.83$, $P=0.02$), leading to increased free baseline and free induced CORT during molt (Fig. 2C; $F_{1,123}=2.8$, $P=0.002$). Molt intensity did not, however, affect the degree of free CORT increase during handling ($P=0.13$). Molt progress had no effect on baseline or induced free CORT ($F_{2,123}=1.9$, $P=0.15$).

Note that if red crossbills were qualitatively grouped as molting vs not-molting (i.e. molt status rather than molt intensity), similar to other molt-stress studies, the results are similar: there are no significant changes in total CORT between groups and free CORT is higher in molting individuals.

Zebra finch location analysis

Though sample sizes were limited, location and molt status interacted to affect the change in total CORT during handling in zebra finches (repeated measures ANOVA: $F_{1,60}=9.3$, $P=0.003$). In the predictable habitat (i.e. Victoria), baseline CORT was lower during molt ($t_{48}=-3.1$, $P=0.003$) whereas in the unpredictable habitat (i.e. Alice Springs), there was a trend for higher baseline CORT during molt ($t_{13}=1.6$, $P=0.12$; Fig. 3). Similarly, there was a trend for lower induced CORT during molt in the Victoria population, and the opposite trend in the Alice Springs population ($t_{49}=-1.6$, $P=0.10$ and $t_{13}=1.8$, $P=0.14$, respectively; Fig. 3).

Molt–CORT comparison across species

Regression analysis revealed a negative relationship between percentage suppression of the HPA axis and molt duration in the 13 species measured to date for both baseline CORT ($F_{1,12}=4.8$, $r^2=0.32$, $P=0.05$) and 30 min CORT ($F_{1,12}=23.1$, $r^2=0.68$, $P=0.0005$) (Fig. 4A). In a preliminary analysis, phylogenetic relationships do not contribute to the observed pattern for 30 min CORT ($F_{1,11}=12.57$,

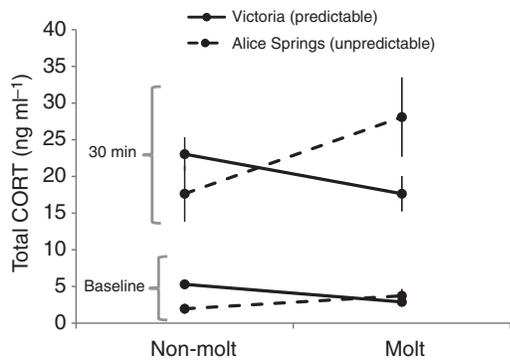


Fig. 3. Difference in CORT levels between non-molting and molting zebra finches in Alice Springs and Victoria. Baseline and maximum CORT both decrease during molt in the more predictable Victoria location ($N=23$ molt, $N=27$ non-molt), but tend to increase in the less predictable Alice Springs location ($N=5$ molt, $N=10$ non-molt). Significant interaction effect of location and molt status in a repeated measures ANOVA ($F_{1,60}=9.3$, $P=0.003$).

$r^2=0.53$, $P<0.05$), but cannot be discounted for baseline CORT ($F_{1,11}=1.76$, $r^2=0.14$, $P=0.10$) (supplementary material Fig. S2B). When the same species were grouped as flexible or seasonal breeders, seasonal breeders showed greater suppression of the HPA axis during molt (baseline CORT $t_{12}=-2.3$, $P<0.05$; 30 min CORT $t_{12}=-5.6$, $P<0.05$) (Fig. 4B).

DISCUSSION

These results demonstrate that two nomadic, opportunistically breeding songbird species do not suppress baseline or induced total CORT levels during plumage molt (Fig. 2A,B). Further, comparative analyses of 13 species reveal negative relationships between molt duration and suppression of the hormonal stress response during molt (Fig. 4). These findings allow a more comprehensive evaluation of hypotheses concerning coordination of self-maintenance processes, and provide an empirical example of how self-maintenance processes may compete for resources to promote survival and affect life-history evolution. We summarize and discuss several of these hypotheses below.

Trade-off hypotheses

There are several hypotheses for the observed molt–stress response relationships that qualify as ‘trade-off’ hypotheses in that they posit competition between molt and the stress response for a limiting resource. Investment of that resource and/or behavioral strategy in one process may only be possible at the expense of the other. These hypotheses include the energy trade-off hypothesis, the molecular trade-off hypothesis and the behavioral trade-off hypothesis.

The energy trade-off hypothesis

Limited energy availability may prohibit simultaneous investment in integument growth and a strong stress response. Molt is energetically expensive and is apparently also rather inefficient (Hoye and Buttemer, 2011; Murphy et al., 1990). This is apparently due to a large degree of body protein turnover, but may also be attributed to reduced thermoregulatory capacity, increased heat loss from blood-filled feather pins exposed to ambient air and reduced flight efficiency (Buttemer et al., 2003; Swaddle and Witter, 1997). Metabolic rates are consistent with these costs and are high during molt (Cyr et al., 2008; Murphy, 1996). The energy demand of a CORT-mediated stress response is more difficult to measure but is

probably high and thus may conflict with feather growth. Consistent with this hypothesis, non-molting starlings subjected to a 30 min restraint stress increased daily energy expenditure by 64%, whereas molting starlings subjected to the same stressor showed no increase in energy cost and secreted only 25% of the maximum CORT levels of their non-molting conspecifics (Cyr et al., 2008). However, metabolic responses to exogenous CORT are variable. For example, if food availability is high, CORT can promote reduced activity and night restfulness (Astheimer et al., 1992; Buttemer et al., 1991). Thus, a CORT-mediated stress response may result in diversion of energy, but may simultaneously promote compensatory responses that minimize energy loss. The energetic consequences of a given response to stressors are inevitably context dependent and therefore highly variable. Environmental predictability and type, duration and severity of environmental stressors should be central concerns in future investigations of the apparent molt–stress trade-off (see also discussion of evolutionary implications below).

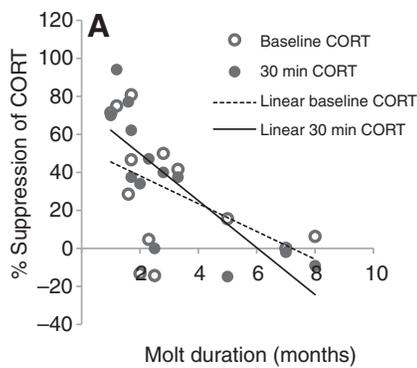
The molecular trade-off hypothesis

Integument replacement requires molecular building blocks, and a classic resource-based trade-off between molt and other protein-demanding processes may occur if those building blocks are in limited supply. Birds’ annual molt can require a quarter or more of a bird’s total body protein mass (Murphy and Taruscio, 1995), much of which comes from muscle tissue (Astheimer et al., 2000; Chérel et al., 1994; Hickson et al., 1995). One of the main effects of elevated circulating CORT is to promote gluconeogenesis, mainly from amino acids, and gluconeogenic effects of increased CORT can cause rapid muscle wastage and protein breakdown (Brown et al., 1992; Busch et al., 2008; De La Cruz et al., 1981). Thus, it has been proposed that suppressed CORT during molt protects protein stores necessary for normal feather growth (Astheimer et al., 1995; Romero et al., 2005). Experimental elevation of CORT through exogenous implants slows feather growth (Romero et al., 2005) and reduces barbicular hooking (DesRochers et al., 2009) in short-duration molters. However, endogenous elevations of CORT *via* exposing birds to psychological or physical stressors have yielded mixed results. Psychological stressors had no effect on feather quality but European starlings subjected to food restriction grew feathers of lower mass in one study (Strochlic and Romero, 2008) and weaker feathers in a second (DesRochers et al., 2009).

The behavioral trade-off hypothesis

Trade-offs may also arise when two processes require or benefit from mutually exclusive behaviors. During molt many birds become highly secretive and more sedentary (Morton and Morton, 1990; Portugal et al., 2007), presumably thereby decreasing exposure to predators, protecting sensitive developing feather follicles and decreasing energy expenditure (Kuenzal, 2003; Murphy, 1996). Although glucocorticoids can reduce activity, they are more often associated with increased activity (Astheimer et al., 1992; Breuner et al., 1998; Breuner and Hahn, 2003; Cornelius et al., 2010; Lohmus et al., 2003; Silverin, 1997). Thus, birds may have evolved a seasonal down-regulation of CORT to suppress active behaviors (Landys et al., 2006). This tactic would be particularly beneficial to species that can access resources in a relatively confined area. Nomadic species, however, may require maintenance of the stress response to assist in dealing with unpredictably fluctuating resources.

Red crossbills specialize on conifer seeds that develop in spatially unpredictable patterns and decline in temporally variable patterns (Koenig and Knops, 2001; Larson and Bock, 1986). It may therefore be necessary for red crossbills to respond maximally to changes in



food availability across the year. Comparative data from other cardueline finches that show a similar slow, seasonal molt but feed on more predictable resources (e.g. greenfinch) may be particularly informative with regard to this hypothesis (Newton and Rothery, 2005). Zebra finches are similarly known to move long distances to locate resources (e.g. water) in the central arid regions of Australia (Zann, 1996); however, they also occur in semi-arid temperate regions of Australia where water and seed availability are much more predictable (Perfito et al., 2007). Despite similar molt durations in the two populations (Zann, 1996), preliminary data suggest that zebra finches in the seasonal temperate habitat show down-regulation of the stress response system during molt whereas zebra finches in the arid interior show a trend to increase stress responsiveness during molt (Fig. 3). This suggests that the degree of suppression may not be strictly related to molecular resources and may be either individually plastic or locally adapted. A similar phenomenon may occur in Eurasian tree sparrows that maintain hormonal stress responsiveness during molt on the Tibetan plateau (Li et al., 2008) but show 50% suppression in lowland populations (Dongming et al., in press).

Non trade-off hypotheses

There are several hypotheses for the observed molt–stress response relationships that are alternatives to the ‘trade-off’ hypotheses (i.e. they do not posit competition between the molt and the stress response for a limiting resource).

Phylogenetic relationships

Preliminary phylogenetic analysis of induced CORT levels suggests that this relationship is not simply a phylogenetic artifact (supplementary material Fig. S2). The analysis, however, includes just 13 species; thus, a broader range of taxonomic groups is certainly required to increase confidence in the relationship and underlying mechanisms. This is especially important given that phylogenetic contributions cannot be statistically rejected for baseline CORT ($P=0.10$) and one species (i.e. the Eurasian tree sparrow) may not fit the expected pattern.

CORT as a molt regulator

There is some evidence that glucocorticoids and the HPA axis play mechanistic or regulatory roles in integument molt. Adenohypophysectomized amphibians fail entirely to engage in their normally cyclic molt (Stefano and Donoso, 1964). Cyclical molt is restored in these animals when they are given cortisol implants (Stefano and Donoso, 1964), but if exogenous cortisol is given to intact animals the molt period is greatly extended (Barker Jørgensen, 1988). In mammals, CORT can be either relatively high or low

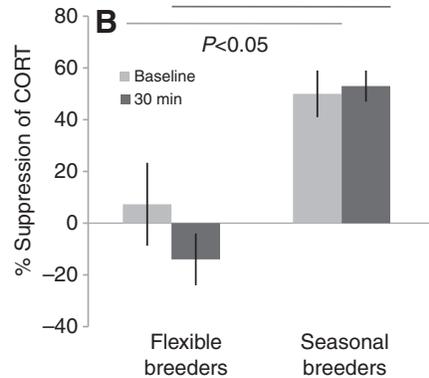


Fig. 4. Multiple species comparison of mean percentage suppression of the HPA axis. There is a negative relationship between molt duration and percentage suppression of the HPA axis (A, baseline CORT $F_{1,12}=4.8$, $r^2=0.32$, $P=0.05$; 30 min CORT $F_{1,12}=23.1$, $r^2=0.68$, $P=0.0005$). Species with a more flexible breeding season (i.e. red crossbill, zebra finch and rock dove) show less suppression than do more seasonal breeders (B, baseline CORT $t_{12}=-2.3$, $P<0.05$; 30 min CORT $t_{12}=-5.6$, $P<0.05$). Molt durations and CORT data were gathered from the literature (Table 1) and percentage suppression was calculated as described in Materials and methods.

during pelage molt, but levels may relate more meaningfully to specific stages of hair shedding vs new growth (Boily, 1996; Riviere et al., 1977; Weiss et al., 1980). It is plausible that CORT has direct influences on the mechanisms involved with integument replacement in birds. For example, CORT implants resulted in slower feather growth in European starlings (Romero et al., 2005). However, repeated acute exposure to CORT delayed molt onset but did not change molt duration in white-crowned sparrows (Busch et al., 2008). The mechanisms underlying molt in birds are still poorly understood and are complicated by other hormones suspected to be important regulators, such as prolactin (Dawson and Sharp, 1998).

Direct effects of CORT

It is also possible that CORT has direct deleterious effects on feather growth, irrespective of growth rate, and that this is responsible for the widespread occurrence of stress response suppression during molt in birds. If true, this would predict that either feather quality is lower in species that maintain elevated CORT during molt or that feather growth is somehow protected from the negative effects of CORT. It is unlikely that crossbills and zebra finches grow low quality feathers as both species require long distance, facultative movements to locate unpredictable resources (Newton, 1972; Zann, 1996). Crossbills, as well as Eurasian tree sparrows, also face significant thermoregulatory demands that must favor high quality insulation (Adkisson, 1996; Hahn et al., 1992). Therefore, if CORT does have direct deleterious effects on feather growth it seems more likely that there is a protective mechanism in taxa that do not suppress CORT secretion during molt. Hypotheses that could explain such protection include that CBG could act either (1) as a buffer against rapid increases in CORT or (2) as a delivery facilitator for CORT. According to the buffer hypothesis, the amount of biologically relevant CORT would be the unbound (i.e. free) CORT; our data do not support the predictions of this hypothesis. Red crossbills do not show the predicted elevation of CBG during molt, but rather a decrease resulting in higher free CORT levels (Fig. 2C,D). These results would therefore be consistent with a protective role only if CBG is acting as a delivery-enhancing molecule. Decreased CBG capacity during molt would reduce the effective delivery of CORT, thereby reducing direct negative effects of CORT on feather growth.

Elevation vs suppression of CORT

Finally, species maintaining the stress response often overlap breeding and molt and do not show large increases in CORT during breeding (Fig. 4) (Perfito et al., 2007) (J.M.C., unpublished data). The observed patterns may therefore be a result of a general aseasonality in stress responsiveness, rather than specific maintenance of CORT during molt. This hypothesis would suggest

that seasonal breeders show hormonal elevation during breeding as opposed to suppression during molt. CORT data prior to breeding (i.e. during winter) are relatively sparse in the literature. Gray crowned rosy finches (Richardson, 2003) and Gambel's white crowned sparrows (Romero et al., 1997) had lower baseline CORT in the winter relative to breeding and molt stages. However, two other seasonally breeding species, the European starling (Romero and Remage-Healey, 2000) and the house sparrow (Romero, 2006), had no differences in baseline or induced CORT between overwintering and breeding and willow warblers had higher CORT in the winter relative to breeding (Silverin, 1998). The existing data are therefore relatively ambiguous in relation to this hypothesis.

Evolution of annual schedules of molt and stress physiology

The molt–CORT trade-off hypotheses all predict that the degree of hormonal suppression is ultimately determined by the relative adaptive values of maintaining the stress response vs the optimal rate of feather growth and/or feather quality.

This conflict should become less severe if competition between processes is minimized by slowing molt or by suppressing CORT-mediated processes. Molt duration is probably constrained by many selective pressures. It is often assumed that molt duration should be minimized because molt decreases thermoregulatory capacity and flight efficiency and increases allostatic load (Buttemer et al., 2003; Hedenstrom and Sunada, 1999; Murphy, 1996). However, prolonging molt and reducing the number of feathers or tissues being replaced at one time can theoretically reduce each of these costs. Species that are constrained to a short molt (e.g. at high latitudes where climate conditions strictly enforce migration departure date) should therefore face a more severe trade-off. Short-duration molt may, therefore, require down-regulation of the hormonal stress response for one or more of the reasons described above. Behavioral and physiological adjustments may help compensate for diminished stress responsiveness. However, if environmental parameters dictate that hormonal stress responsiveness must be maintained during the molt period, then slower feather growth may be required to preserve feather quality. Nomadic species dealing with unpredictable resources (e.g. zebra finches and red crossbills) may exemplify this trade-off. Alternatively, if molt duration is increased in response to other selective pressures (e.g. increased flexibility for timing of breeding; Fig. 4), then maintenance of the hormonal stress response may simply be allowed because resource demands are spread further across time.

Significance

Relative contributions of conflicting self-maintenance processes to fitness may affect the evolution of CORT-mediated responses to stress or integument replacement. This may have important implications for species persistence as selective pressures shift with environmental change. Migratory routes and timing have shifted recently in some species, and this may exert pressure for molt scheduling to change as well (Van Der Jeugd et al., 2009; Walther et al., 2002). Species may simultaneously face increased exposure to stressors as a result of unfamiliar or shifting environmental conditions. The effects of environmental change on species persistence may therefore depend, in part, on the plasticity – or constraints thereon – inherent to conflicting self-maintenance processes.

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