RESEARCH ARTICLE

Effects of Immune Activation and Glucocorticoid Administration on Feather Growth in Greenfinches

MARJU MÄNNISTE AND PEETER HÕRAK*
Department of Zoology, Institute of Ecology and Earth Sciences, Tartu University, Tartu, Estonia

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ABSTRACT

Elevation of glucocorticoid (GC) hormone levels is an integral part of stress response (as well as its termination) and immunomodulation. These hormones are also responsible for mobilizing energy stores by stimulation of gluconeogenesis and inhibition of protein synthesis. Elevation of GCs is thus incompatible with other protein-demanding processes, such as moult. Previous studies have shown that chronic elevation of GC hormones suppresses feather growth. Here, we asked whether similar effect would also occur in the case of acute GC elevation and induction of an inflammatory response by foreign antigen. We performed an experiment on captive wild-caught greenfinches (*Carduelis chloris*) injecting birds with phytohaemagglutinin (PHA) and dexamethasone (DEX) in a factorial design. To assess the possible somatic impacts of these manipulations, we removed one of the outermost tail feathers before the experiment and measured mass and rachis diameter and length of the replacement feathers grown in captivity. Immunostimulation by PHA reduced rachis length, but did not affect feather mass or rachis diameter. Single injection of a synthetic GC hormone DEX significantly reduced all three parameters of feather size. Altogether, these findings demonstrate the sensitivity of feather growth to manipulation of immune and adrenal functions. Our results corroborate the somatic costs of immune activation and suggest that even a short-term elevation of GC hormones may induce long-term somatic costs with a potential impact on fitness. Our findings also imply that a single injection of DEX, frequently used as a diagnostic tool, can have lasting effects and researchers must consider this when designing experiments. *J. Exp. Zool. 313A, 2011.* © 2011 Wiley-Liss, Inc.

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Immune defences belong to the most complicated and resource-demanding organismal functions (Schulenburg et al., 2009). Detection and destruction of parasites and pathogens involves construction of sophisticated recognition and memory pathways, release of harmful substances and increased metabolism. All those impose costs, which eventually lead to physiological trade-offs in allocation of resources between immunity and other components of fitness. A nascent discipline—immunoecology—proposes that such trade-offs have major impact on the evolution of physiological and life-history strategies (Sheldon and Verhulst, '96; Lochmiller and Deerenberg, 2000; Lee, 2006; Schulenburg et al., 2009). With the advancement of immunoecology, the initially naïve contention of many animal ecologists that immunocompetence can be considered as an ubiquitous commodity (the stronger the response, the better) has been gradually replaced with the appreciation of the importance of proper downregulation and termination of immune responses (Råberg et al., '98; Graham et al., 2005, 2010; Day et al., 2007; Martin, 2009; Sorci and Faivre, 2009). Those conceptual advancements, however, have not yet been caught up with sufficient empirical work.

Important role in downregulation and termination of the immune responses belongs to glucocorticoid (GC) hormones (Perretti and D'Acquisto, 2009), cortisol (in humans), or

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*Correspondence to: Peeter Hõrak, Department of Zoology, Institute of Ecology and Earth Sciences, Tartu University, Vanemuise 46, 51014 Tartu, Estonia. E-mail: horak@ut.ee
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corticosterone (CORT; in rodents, amphibians, reptiles, and birds). GC hormones induce immunosuppression through multiple transcriptional mechanisms (Sapolsky et al., 2000) as well as more rapid, nongenomic effects on cellular responses (Löwenberg et al., 2007). Besides immunomodulation, baseline GC hormone levels are considered crucial in mobilizing energy stores for the fight-or-flight response. This occurs via inhibition of protein synthesis and mobilization of endogenous glucose stores (Remage-Healey and Romero, 2000). It is believed the GC-induced increase in energy levels sustains the period of heightened alertness following a stressor, allowing an animal to recover from a stimulus, and better respond to a subsequent stressor (Sapolsky et al., 2000). Importantly, switching off the stress response is based on the negative feedback by GC hormones (Sapolsky et al., 2000). Functioning of this switch is often studied by administration of a synthetic corticosteroid dexamethasone (DEX), which decreases circulating endogenous CORT titres if feedback is functioning normally (e.g., Dickens et al., 2009b; Romero and Wikelski, 2010).

A suitable model to study catabolic effects of corticosteroids is avian moult. It was first suggested by Romero et al. (2005) that birds downregulate CORT release during moult in order to avoid the protein catabolic activity of CORT from directly inhibiting the protein deposition necessary to produce feathers. In experiments on adult starlings (*Sturnus vulgaris*) where baseline CORT levels were chronically increased, CORT-implanted birds showed a significantly decreased rate of feather growth (Romero et al., 2005; Strochlic and Romero, 2008; DesRochers et al., 2009). Similar pattern was observed in nestling American kestrels (*Falco sparverius*) (Butler et al., 2010). In nestlings of altricial birds, even a short-term (2–3 days) upregulation of CORT can suppress the growth of feathers, bones, and body mass, as demonstrated in Eurasian kestrels (*Falco tinnunculus*) by Müller et al. (2009).

Here, we ask whether a single, short-term elevation of GC hormone levels can also affect feather growth in adult birds. To put this information into wider ecophysiological context, we tested whether such potential somatic cost is of comparable magnitude with the one induced by immune system activation. Both immune and stress responses deplete somatic resources; however, the question about which of those is more expensive has never been directly assessed. For instance, in captive greenfinches, single GC administration and immune activation by a toxic plant lectin phytohaemagglutinin (PHA) induced similar increase in the concentration of circulating heterophils (Sepp et al., 2011). However, a hematological stress index, the heterophil/lymphocyte ratio, was affected only by the GC treatment but not by immune challenge. We were, thus, interested whether these immune activation and GC administration treatments would have similar effects on feather growth as well. Such information is also relevant for immunoecological and stress research, in general. For instance, termination of both immune and stress responses involves increasing the levels of GC hormones (Sapolsky et al., 2000). To account for the net effects of altered corticosteroid levels, one should be aware of the relative magnitude of costs induced by both activation and suppression of stress and immune responses. This kind of research is important, especially given that the questions when, how, and why stress and immunity interact in wild animals remains poorly understood (e.g., Martin, 2009). Finally, assessment of the effects of immune activation and GC administration on different parameters of feather growth also enables to find out which of those parameters are most sensitive to such treatments. This information is of methodological interest for studies aiming to quantify somatic impacts of physiological manipulations in birds.

To add further ecological dimension to the study, we also asked whether supplementation of dietary carotenoids affects the possible somatic cost imposed by immune activation and immune suppression. Carotenoid-based signals have been in the scope of extensive research by animal ecologists since recognition of their possible role in health maintenance and signaling. Carotenoids can modulate immune function (Chew and Park, 2004) and may also act as antioxidants (Krinsky, '89; but see Costantini and Møller, 2008). As they must be acquired from food and are destroyed when used as antioxidants (Vershinin, '99), it is believed that carotenoids are limited for use as colorants in ornaments, such as feathers, skin, and scales. Therefore, it has been suggested that these visual characters enable individuals to convey honest information on their bearers' phenotypic and/or genetic quality to potential mates and opponents (Lozano, '94). So far, few studies have shown that carotenoid supplementation can attenuate body mass loss imposed by immune challenge (Hõrak et al., 2006; Pap et al., 2009; Sepp et al., 2011) and that CORT administration can interfere with carotenoid sequestration to integument (Loiseau et al., 2008; Cote et al., 2010; but see Costantini et al., 2008). We, therefore, predicted that carotenoid supplementation will positively affect feather growth of birds receiving immune activation and GC challenges.

To study these questions, we performed an experiment with captive wild-caught greenfinches (*Carduelis chloris*). Greenfinches are about 30 g sexually dichromatic seed-eating passerines, which are becoming model objects in immunoecological research (e.g., Peters et al., 2008; Hõrak et al., 2010). In this experiment, we performed GC administration and immune activation treatments, injecting birds with PHA and synthetic GC DEX in a factorial design. Admittedly, using a synthetic GC instead of CORT has its drawbacks. Although DEX is an agonist of CORT and binds to the CORT receptors, it is not identical to CORT (reviewed by Holberton et al., 2007). We used DEX instead of CORT because the latter is not soluble in saline and the use of organic diluents, such as DMSO or ethanol, can have independent effects on CORT responses (Busch et al., 2008) and immunity (Post et al., 2004). Such trade-offs in choosing the research

methodology are inevitable in GC manipulations (see also Strochlic and Romero, 2008). To assess the possible somatic impacts of these manipulations, we removed one of the outermost tail feathers before the experiment and measured the mass, length, and rachis diameter of the replacement feathers grown in captivity.

PHA assay is the most popular standard test for assessment of the immune function in wild animals. PHA is an anti-herbivore plant lectin which is toxic to animals (Vasconcelos and Oliveira, 2004). Subcutaneous injection of PHA induces T-cell mitogenesis and produces a localized swelling response involving local infiltration of tissue by most types of immune cells. The magnitude of this swelling has been interpreted to reflect both acquired T-cell-mediated immunocompetence (Tella et al., 2008; but see Vinkler et al., 2010) and nonspecific basophile-mediated inflammation (Martin et al., 2006a). Immune response to PHA elevates metabolic rate in some species (Martin et al., 2003; Nilsson et al., 2007), but decreases it in others (Lee et al., 2005). Because inflammation is considered the most expensive aspect of immune activation (Klasing, 2004; Schmid-Hempel, 2008; Sorci and Faivre, 2009), PHA injection provides an opportunity for assessment of the physiological costs of mounting immune response. For instance, PHA injections can change the levels of reactive oxygen metabolites, antioxidant capacity, carotenoids, and nitric oxide metabolites in the blood of birds (Costantini and Dell'Omo, 2006; Hõrak et al., 2007; Perez-Rodriguez et al., 2008; Sild and Hõrak, 2009). Importantly, PHA injection can hinder feather growth (Martin, 2005).

DEX is a CORT agonist which binding on the same receptors can decrease endogenous CORT production via negative feedback on the hypothalamic–pituitary cascade at the level of the pituitary (reviewed by Holberton et al., 2007). DEX injections are routinely used for immune suppression (Huff et al., '99) and manipulation of CORT levels (Rich and Romero, 2005). In greenfinches, a single injection of DEX in physiological dose significantly reduced PHA-induced swelling response and body mass (Sepp et al., 2011).

METHODS

Male greenfinches ($N = 93$) were caught in mist nets in the Sõrve Bird Observatory on the island of Saaremaa (57°55′N; 22°03′E) from January 25 to 27, 2007. Birds were transported to Tartu and housed indoors in individual cages (27 × 51 × 55 cm) with sand bedding. Average temperature in the aviary during the experiment was 15.9 ± 1.7°C (SD) and average humidity was 53.2 ± 2.6% (SD). The birds were supplied ad libitum with sunflower seeds and filtered tap water. Birds were held on the natural day-length cycle on artificial lighting, increasing continuously the length of the light period from 8 to 13 hr by the end of the study. The birds were released into their natural habitat on April 1. The left outermost tail feather was plucked from all of the birds on February 8 and the replacement feather, grown in captivity, was collected on April 1, i.e., after 53 days. Both wild-grown and lab-grown feathers were weighed with a Mettler Toledo electronic balance (Schwerzenbach, Switzerland) (model XP26) with a precision of 0.1 mg. The dorsoventral diameter of the rachis was measured to the nearest 0.01 mm at the basis of vane with a spessimeter (SM112, Teclock, Japan). Repeatability (Lessells and Boag, '87) of rachis diameter, based on the triplicate measurement of a subsample of feathers, was 0.94 ($F_{9,10} = 45.1$; $P < 0.00001$). Length of the extended feathers (i.e., rachis length) was measured with a ruler with an accuracy of 0.5 mm. Repeatability of feather length was 0.99 ($F_{9,10} = 1{,}150$; $P < 0.00001$). The study was conducted under the license from the Estonian Ministry of the Environment and the experiments comply with the current laws of the Estonian Republic.

On February 6, birds were divided into three treatment groups, which were set to have similar average body mass at capture and age composition. Thirty-three birds started to receive high doses of carotenoid supplementation, 32 birds received low doses of carotenoids, and 28 birds (controls) received filtered tap water. Supplementation consisted of 18 (high dose) or 6 (low dose) µg/mL water solution of lutein and zeaxanthin (20:1, w/w), prepared from OroGlo liquid solution of 11 g/kg xanthophyll activity (Kemin AgriFoods Europe, Herentals, Belgium). Those solutions were freshly prepared each evening using filtered (Brita® Classic; BRITA GmbH, Taunusstein, Germany) tap water and were provided in 30 mL doses in opaque dispensers in order to avoid oxidation of carotenoids. Carotenoid supplementation lasted 19 days.

In the evening of February 24, birds were assigned to 2 × 2 treatments of immune challenge and DEX administration. Half the birds from each treatment group were injected intradermally in the wing web with 0.2 mg of PHA (Sigma, St. Louis, MO; L-8754) in 0.04 mL of sterile isotonic saline. Similar doses are commonly used in small passerines (Lifjeld et al., 2002; Martin et al., 2006b; Palacios and Martin, 2006). At the same time, the rest of the birds were injected with saline. These treatment groups were again split into half, so that half the group received an injection of DEX. DEX (KRKA, d.d., Novo Mesto, Slovenia; 0.03 mg in 0.05 mL sterile isotonic saline; set to approximate 1 mg/kg body weight) was injected into pectoralis muscle while the rest of the birds received the same amount of isotonic saline injection. The dose of DEX was chosen on the basis of calculations by Remage-Healey and Romero (2001) as to mimic the natural stress-induced elevation of CORT in small passerines. DEX injection took place immediately after PHA or saline injection into wing web. All birds were blood sampled on February 21 and 27 and March 26 for other research purposes (Sarv and Hõrak, 2009; Sepp et al., 2011).

Effects of experimental treatments upon the mass of lab-grown feathers were assessed in factorial ANCOVAs, keeping the mass or size parameters of wild-grown feathers as covariates. We used two-tailed tests with an α-level below 0.05 as a criterion for

significance. Our final sample size was 71, because some of the birds had lost their replacement feathers before these became full-grown and because some feather tips were worn off, precluding measurement of feather length. Wild-grown feathers were always in perfect condition (see Fig. 1 in Sild et al., 2011).

RESULTS

Correlations between different parameters of feather size were mostly significant but not particularly strong (Table 1). DEX injection significantly reduced all three measures of feather size, whereas PHA injection had a significant effect on feather length only (Table 2 and Fig. 1). After adjusting for the size of

Table 1. Pearson correlations (*P*-values in parentheses) between different parameters of wild- and lab-grown feathers of greenfinches ($N = 71$).

	Mass (lab)	Length (wild)	Length (lab)	Diameter (wild)	Diameter (lab)
Mass (wild)	0.64 (0.002)	0.47 (<0.0001)	0.47 (<0.0001)	0.21 (0.081)	0.26 (0.030)
Mass (lab)		0.52 (<0.0001)	0.68 (<0.0001)	0.36 (0.002)	0.50 (<0.0001)
Length (wild)			0.73 (<0.0001)	0.22 (0.064)	0.29 (0.014)
Length (lab)				0.17 (0.153)	0.19 (0.119)
Diameter (wild)					0.51 (<0.0001)

Table 2. Effects of DEX, PHA, and carotenoid (CAROT) treatments on the mass, length, and rachis diameter of replacement feathers grown during the experiment.

Dependent variable	Predictors	df	*F*	η^2	*P*
Lab-grown feather mass	Wild-grown feather mass	1,58	33.3	0.36	<0.00001
	DEX	1,58	6.2	0.10	0.015
	PHA	1,58	0.3		0.594
	CAROT	2,58	0.0		0.807
	DEX∗PHA	1,58	0.0		0.571
	CAROT∗DEX	2,58	1.6		0.205
	CAROT∗PHA	2,58	1.5		0.235
	CAROT∗DEX∗PHA	2,58	0.9		0.418
Lab-grown feather length	Wild-grown feather length	1,58	52.7	0.48	<0.00001
	DEX	1,58	11.3	0.16	0.001
	PHA	1,58	5.2	0.08	0.026
	CAROT	2,58	1.3		0.273
	DEX∗PHA	1,58	0.1		0.815
	CAROT∗DEX	2,58	0.6		0.526
	CAROT∗PHA	2,58	0.2		0.853
	CAROT∗DEX∗PHA	2,58	1.4		0.254
Lab-grown rachis	Wild-grown rachis	1,58	20.3	0.26	<0.00001
	DEX	1,58	4.9	0.08	0.031
	PHA	1,58	0.0		0.987
	CAROT	2,58	0.1		0.104
	DEX∗PHA	1,58	0.0		0.934
	CAROT∗DEX	2,58	0.1		0.911
	CAROT∗PHA	2,58	0.6		0.570
	CAROT∗DEX∗PHA	2,58	0.5		0.613

η^2 stands for coefficients of partial determination, describing the proportion of total variation attributable to the factor, partialling out other factors from the total nonerror variation. See Figure 1 for sample sizes.

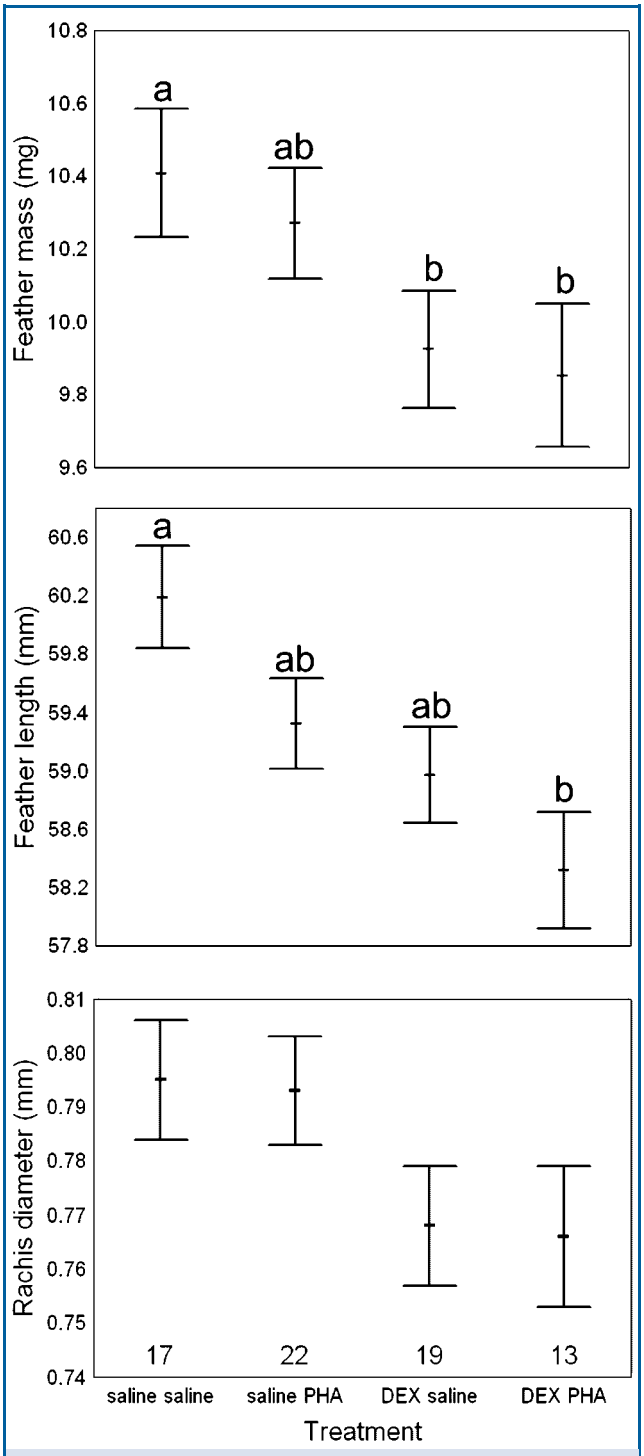

Figure 1. Effects on the PHA and DEX treatments on the mass, length, and rachis diameter of replacement feathers of greenfinches, grown during the experiment. Least square means ± SE from the models adjusting for initial (wild grown) feather mass. Numbers indicate sample sizes. Averages marked with different letters are significantly different from each other (Tukey HSD test for unequal sample sizes).

wild-grown feathers, the feathers of DEX-injected birds were on average 4% lighter than those of the birds not receiving DEX (9.89 ± 0.13 vs. 10.34 ± 0.11 mg); they were also 2% shorter (58.6 ± 2.6 vs. 59.7 ± 2.3 mm) and had 3% thinner rachis (0.77 ± 0.01 vs. 0.79 ± 0.01 mm; all LS means ± SE from the models in Table 1). PHA injection reduced feather length on average by 1% (58.8 ± 2.5 vs. 59.6 ± 2.4 mm). Coefficients of partial determination indicate that 8% of variation in lab-grown feather length was explained by the PHA treatment and 16% by the DEX treatment. Carotenoid treatment had no effect on any parameters of feather size (Table 2).

DISCUSSION

This study clearly showed that a single boost of GC hormone is sufficient to restrict feather growth of wild birds, despite the benign conditions and ad libitum access to food in captivity. The effect of DEX injection was detectable on all three measured parameters of feather size. These parameters reflect somewhat different components of feather quality as correlations between them were not particularly strong (Table 1). Developmental pathways affecting these parameters may not be identical. For instance, Sillanpää et al. (2010) found that fluctuating asymmetry in great tit (*Parus major*) tail feather length and mass was affected by different dietary and pollution-related factors, and that asymmetry in these two morphological characters was only weakly correlated. The functional relationships between our measures of feather size and quality are also diverse. Feather mass has been shown to correlate with number of barbs, and hence the tightness of feather surface (Butler et al., 2008). Feather tightness enhances heat absorption and sustained flight (Butler et al., 2008) and resistance to abrasion (Dawson et al., 2000). Feather length affects take-off speed and manoeuvrability (Swaddle et al., '96; Matyjasiak et al., 2004), and hence predator escape. Rachis diameter affects rigidity of feather (Dawson et al., 2000). Thus, all the factors affecting moult have direct impact on fitness as reduction in plumage quality can influence survival by decreasing flight performance and increasing thermoregulatory costs (Nilsson and Svensson, '96; Swaddle et al., '96; Dawson et al., 2000; DesRochers et al., 2009). Indirectly, plumage quality can impinge on fitness through the process of mate choice (e.g., Pryke and Andersson, 2005). The latter implies that feather size contains information about the ability of its bearer to avoid stressful situations or block the catabolic effects of CORT during stress.

Finding that a single DEX administration suppressed feather growth has also practical implications for studies measuring the negative feedback capacity of Hypothalamic–Pituitary–Adrenal (HPA) axis to downregulate CORT levels in response to DEX injection. Such protocols are well established (reviewed by Dickens et al., 2009a). This study indicates that when DEX injections are used in studies monitoring changes in individual condition, it would be important to consider carefully the

possible somatic costs accompanying the physiological effects of DEX.

We can see two mutually nonexclusive pathways of how DEX injection can affect feather growth. First, immune suppression by DEX could have increased susceptibility of greenfinches to opportunistic infections, with accompanying metabolic costs. In the same experiment, we found no systematic effects of DEX on the development of chronic coccidian infection (Sepp et al., 2011); however, we cannot exclude the possibility of facilitation of relapse of other microbial infections. Second, the effects of DEX on feather growth might be owing to GC-induced suppression of protein synthesis (see Sapolsky et al., 2000). Unfortunately, this experiment cannot differentiate between these two possibilities.

Previous studies have established the suppressive effects of chronic CORT administration on feather growth (Romero et al., 2005; Busch et al., 2008; Strochlic and Romero, 2008; Butler et al., 2010). Here, we used acute administration of DEX in a dose that was set to mimic an effect of GC release during an episode of acute stress. We proceeded from the calculations by Remage-Healey and Romero (2001) in starlings where an injection of CORT in a dose of ca 1 mg/kg body weight increased of CORT levels from 40 to 50 ng/mL, which is comparable to CORT induced by 15–45 min of restraint stress (Romero and Remage-Healey, 2000). Admittedly, our treatment did not fully mimic the endogenous surge of CORT, as DEX has different affinity and specificity to corticosteroid-binding globulins than CORT (reviewed by Holberton et al., 2007). However, the possibility that our DEX treatment mimicked the catabolic effect of endogenous elevation of CORT levels cannot be excluded. In further studies, it would be most interesting to evaluate whether natural downregulation of HPA axis by negative feedback mechanism can also induce similar somatic costs to an exogenous DEX administration.

PHA injection affected only one of the three measures of feather size, the feather length. Thus, immune activation had less effect on the total amount of protein allocated into feathers than on arrangement of this protein for building up the feather structure. Such pattern could emerge either owing to developmental constraints or owing to different investment priorities. The latter scenario would imply that, in the face of resource limitation, investment into feather mass (which increases feather strength) is prioritized over investment into extension of feathers. Varying effects of CORT on different feather parameters were also documented by DesRochers et al. (2009) in starlings. Interestingly, in starlings, exogenously administered CORT reduced mass but did not affect the length of retrices, which is exactly the opposite of current findings in greenfinches.

We did not detect any significant interactions between DEX and PHA treatments in ANCOVAs, describing predictors of feather size (Table 2). In the case of feather length, the effects of both treatment were additive and in the case of feather mass and rachis diameter, the effect of DEX was independent of PHA (Fig. 1). We found, thus, no evidence that suppression of swelling response to PHA by DEX had spared any resources for enhancement of feather growth.

The effect of PHA injection on feather length compares favourably with several experiments in wild birds showing that induction of immune responses against novel antigens inhibits or delays moult (Ilmonen et al., 2000; Sanz et al., 2004; Martin et al., 2005; Moreno-Rueda, 2010; but see Pap et al., 2008). Similar to this study, Martin et al. (2005) injected PHA into house sparrows (*Passer domesticus*) and found that immune challenge significantly reduced the number of growing flight feathers 3 weeks after injection. PHA induces a complex inflammatory response which involves many different immune components (reviewed by Sarv and Hõrak, 2009; Vinkler et al., 2010). This study adds another piece of evidence about somatic costs of such induced responses in terms of feather growth. Importantly, this study also exemplifies the value of measuring several parameters of feather size. For instance, we would not have detected the impact of immune activation by measuring the feather mass alone.

Previous studies in greenfinches have shown that carotenoid supplementation can incline birds to fattening and alleviate the body mass loss induced by immune challenge with sheep erythrocytes (Hõrak et al., 2006) or PHA (Sepp et al., 2011). Carotenoid-supplemented house sparrows lost less mass after experimental coccidian infestation than unsupplemented sparrows (Pap et al., 2009). Current experiment showed that dietary carotenoids had no effect on feather growth. This suggests that any possible anabolic effects of carotenoids involve lipid rather than protein metabolism.

In conclusion, we have shown that a single injection of DEX, set to mimic a natural elevation of CORT in response to acute stress, significantly reduced the size of replacement feathers in captive wild-caught greenfinches. This raises the possibility that experiencing an acute stress or termination of immune responses by elevation of GC hormones may induce long-term somatic costs with a potential impact on fitness. We also detected an effect of PHA-induced inflammatory response on feather length, which corroborates the somatic costs of immune activation. Altogether, these findings demonstrate the sensitivity of feather growth to manipulation of immune and adrenal functions, which reinforces the potential utility of feather parameters for assessment of somatic impacts of physiological manipulations in birds.

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