

## Physiological, morphological and behavioural effects of selecting zebra finches for divergent levels of corticosterone

M. L. Roberts<sup>1,\*</sup>, K. L. Buchanan<sup>2</sup>, D. Hasselquist<sup>3</sup>, A. T. D. Bennett<sup>4</sup> and M. R. Evans<sup>1</sup>

<sup>1</sup>Centre for Ecology and Conservation, School of Biosciences, University of Exeter, Cornwall Campus, Penryn, Cornwall TR10 9EZ, UK, <sup>2</sup>School of Biosciences, Main Building, Park Place, Cardiff University, Cardiff CF10 3TL, UK, <sup>3</sup>Department of Animal Ecology, Lund University, Ecology Building, Sölvegatan 37, 22362 Lund, Sweden and <sup>4</sup>School of Biological Sciences, Woodland Road, University of Bristol, Bristol BS8 1UG, UK

\*Author for correspondence at present address: Max Planck Institute for Ornithology, Vogelwarte Radolfzell, Schlossallee 2, 78315 Radolfzell, Germany (e-mail: roberts@orn.mpg.de)

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### Summary

The effects of environmental stress on the physiology and behaviour of higher vertebrates has become an important avenue of research in recent years. Evidence from recent studies has suggested that the avian stress-related hormone corticosterone (CORT) may play a role in immunocompetence and sexual selection. We tested whether CORT is immunosuppressive by studying humoral and cell-mediated immune responses in populations of captive zebra finches selected for divergent peak levels of CORT. We also investigated whether selection for peak CORT has an effect on the quality of several sexually selected regions of the male zebra finch; in addition we compared morphometric parameters and the dominance ranking in males from the different selection lines. We also tested whether different components of the immune system compete for limited resources. We found that selection for divergent levels of peak CORT had little effect on humoral immunity, male sexual signal quality or

dominance ranking. However, contrary to expectations, we did find a positive relationship between CORT titre and cell-mediated immunity, as well as a greater cell-mediated response in the birds selected for high CORT titre than those selected for low CORT titre. Consistent with predictions, significant negative relationships were found between both testosterone and CORT titre on humoral immunity. Birds from the low CORT lines were significantly larger in terms of skeletal size than those from the high CORT lines. Overall, our results suggest that the cell-mediated immune response is associated with a reduction in the humoral response, but only in males, and that there is no simple relationship between peak CORT levels and immune function.

Key words: corticosterone, glucocorticoid, zebra finch, selection experiment, stress, dominance, immunity, skeletal size, sexual signals, testosterone.

### Introduction

The stress response is an important component of an individual's suite of physiological and behavioural responses to environmental events. As such, research into both the physiological consequences of stress and the evolutionary constraints imposed by the response to stress have recently become significant areas of research. The commonly used surrogates of stress are the glucocorticoid steroids produced by the adrenal cortex. These hormones (primarily cortisol in primates, and corticosterone in birds, reptiles and rodents) are released in response to stressful environmental events, and are crucial in initiating the 'flight or fight' response as well as a range of essential physiological and behavioural reactions to stress (Nelson, 1995). These include increasing the peripheral blood supply, reducing digestive processes and mobilising glucose reserves (Silverin, 1998; Buchanan, 2000; Sapolsky et al., 2000); as well as reducing reproductive activity, and increasing both foraging and escape behaviour (Wingfield et al.,

1997; Silverin, 1998; Breuner et al., 1998; Löhmus et al., 2006). Glucocorticoids are pulsatile in nature, and can increase dramatically above a baseline (basal) level within a few minutes of a stressful event (the peak stress response). The difference in the two levels of the steroid is important, as different receptors are involved in baseline and acute (peak) glucocorticoid activity (see Buchanan, 2000; Romero, 2004).

Although the stress response is an adaptive method of dealing with potentially damaging environmental events or conditions (Silverin, 1998), chronically high blood plasma levels of corticosteroids can have deleterious effects on several components of an individual's fitness. For example, there is a wealth of evidence from lab-based studies in small mammals that corticosterone (CORT) is immunosuppressive at high levels (Harvey et al., 1984; Sapolsky et al., 2000; Neigh et al., 2004; Quintanar-Stephano et al., 2004), and there is some evidence from both field and laboratory studies in reptiles and birds that supports this (Berger et al., 2005; Martin et al., 2005; Wingfield

et al., 1997; Råberg et al., 1998). However, the issue of whether CORT is always immunosuppressive is by no means satisfactorily concluded, because a few studies have found either no relationship between CORT and immunocompetence (Saino et al., 2002) or even an immuno-enhancing effect (Svensson et al., 2002). The relationship between CORT and immune function is known to be complex and non-linear, and, crucially, any relationship between immune function and CORT will depend on whether baseline or acute CORT levels are measured. The question remains as to whether corticosteroids directly cause immunosuppression, whether they indirectly cause immunosuppression or whether indirect effects are caused through confounding effects, if individuals with genes for elevated corticosteroid production also have poor immune responses. Furthermore, as yet the influence of heritable variation in CORT production on immune function remains untested.

We also tested whether CORT has a deleterious effect on avian male sexual signals and morphology. There is some evidence to suggest that high levels of CORT may have a negative effect on body condition in birds (Schwabl, 1995; Hood et al., 1998; Kitaysky et al., 2001; Sockman and Schwabl, 2001; Perfito et al., 2002; Breuner and Hahn, 2003; Pereyra and Wingfield, 2003), and this may affect the quality of male sexual signals. This negative relationship between sexual signal quality and CORT level has been found previously (see Saino et al., 2002). However, little further work has been carried out to test whether stress (measured as peak CORT level) affects male visual sexual signalling; nevertheless, a link has been found between developmental stress and song performance in songbirds (Nowicki et al., 2000; Spencer et al., 2003) and CORT and song in toads (Leary et al., 2006). If a connection exists between sexual signal quality and stress then this has important implications for female mate choice and ultimately for the adaptive value of the stress response. Previous work suggests that CORT may adversely affect early development and growth (e.g. Lin et al., 2006), so we also compared body mass and skeletal size between the selection lines.

Finally, we also investigated whether any dominance ranking existed between individuals based upon peak plasma CORT levels. Some studies in birds have found high ranking males to have high levels of both baseline CORT (Mateos, 2005) and peak CORT (Pravosudov et al., 2003; Poisbleau et al., 2005), whereas other studies have found the opposite to be true (Nunez de la Mora et al., 1996), while another set of studies have concluded that there is no relationship between rank and CORT (Schoeche et al., 1997; Parker et al., 2002). If any relationship did exist between CORT level and dominance ranking then this would have implications for theories of social stress and dominance (for a review, see Creel, 2001).

We also measured plasma testosterone levels in the males and included the results in the subsequent statistical models to take into account any confounding effect this hormone may have exerted, particularly on the dominance trials (Wiley et al., 1999), immune tests (Roberts et al., 2004) and sexual signal measurement (McGraw et al., 2006). Some studies have also found significant covariation between CORT and testosterone (e.g. Evans et al., 2000), so taking into account testosterone

levels was crucial in this study to be able to conclusively identify CORT as the effector hormone.

The relationship between CORT, immune function and the production of sexual signals is likely to be a complex one. It is well established that peak CORT levels have a heritable component as two separate selection studies have found similar levels of heritability (Evans et al., 2006; Satterlee et al., 2000). Here, to ascertain whether individuals with heritably different levels of peak CORT have differences in immunocompetence, male sexual signal quality, dominance and skeletal size, we used selected lines of zebra finches (*Taeniopygia guttata*) that have been selected for divergent levels of peak CORT production over several generations (Evans et al., 2006). Previous studies on zebra finches from these selected lines have demonstrated that peak CORT may affect mate choice (Roberts et al., 2007a); spatial memory and mineralocorticoid receptor mRNA expression (Hodgson et al., 2007); personality traits (Martins et al., 2007); and, in conjunction with testosterone manipulation, immune response (Roberts et al., 2007b). This species serves as an excellent model, since it breeds readily in captivity throughout the year, is robust and is sexually dimorphic. In addition, many studies have used the zebra finch as a laboratory model in tests of immunity (Alonso-Alvarez et al., 2004; Birkhead et al., 1998; McGraw and Ardia, 2003; McGraw and Ardia, 2004; McGraw and Ardia, 2005; Snoeijs et al., 2005; Verhulst et al., 2005); sexual signalling (e.g. Alonso-Alvarez et al., 2004; Bennett et al., 1996; Birkhead et al., 1998; Birkhead et al., 1999; Burley and Coopersmith, 1987; Hunt et al., 1997; McGraw and Ardia, 2003); and, to a lesser degree, dominance rank (Beauchamp, 2000; Cuthill et al., 1997). Avian selection experiments have in the past been useful in exploring relationships between sexual selection and components of the immune system (Verhulst et al., 1999).

## Materials and methods

### *Selection programme*

Since 1999 we have selected three lines of zebra finch (*Taeniopygia guttata castanotis* Gould 1837) with two replicates for each (resulting in 6 lines in total) for contrasting levels of peak (i.e. acute) CORT (low, control and high CORT levels) in response to a mild stressor [20 min holding in a cloth bag (see Evans et al., 2006)]. The selection regime and life history consequences are described in detail elsewhere (Evans et al., 2006). There has been a significant difference in plasma CORT levels between the lines in the desired directions since the F2 generation of selection, with selection pressure exerted on each generation (Evans et al., 2006). There has been a downward trend in CORT over the generations regardless of selection line; little difference has existed in changes in CORT titre between the low lines and controls, but the high lines have shown a significant realised heritability of 20–25% and have generally had peak CORT plasma levels three times higher than the other lines (Evans et al., 2006). No corresponding change in testosterone over the generations has been evident, and no significant differences exist in testosterone titre between the CORT lines (Evans et al., 2006). Birds in each line were housed together in a large aviary giving *ca* 1 m<sup>3</sup> per breeding pair and maintained at an ambient temperature of 20–24°C (Jones and Slater, 1999). The humidity was maintained between 50% and

70% and the rooms sprayed with water two or three times per day. The birds were provided with *ad libitum* seeds (foreign finch mixture, Haiths Ltd, Cleethorpes, Lincs, UK), Chinese millet sprays, mineralised grit, water and cuttlefish bone. The finches were provided with *ca* 10 g of a 3:1 mixture of nectarblend (Haiths Ltd) and egg biscuit food (Haiths Ltd), and either lettuce or cucumber daily with a cod liver oil supplement in the seed weekly. An excess of nesting baskets and boxes was provided. Full details of the selection methodology, characteristics and housing can be found in Evans et al. (Evans et al., 2006). We have taken every care to minimise any welfare concerns of this project, and all work was carried out under licence by the UK Home Office (PPL 60/2584) and after local ethical review.

#### *Hormone sampling and assay characteristics*

Blood samples for CORT (100  $\mu$ l) were taken from the brachial vein after 20 min holding in a cloth bag, after a pilot study revealed that peak CORT response occurs in the zebra finch after 20 min restraint (Evans et al., 2006). This is the standard capture–restraint protocol used in studies of the CORT response (Wingfield, 1994). The blood was centrifuged at 11 000 g for 15 min and the plasma frozen at  $-20^{\circ}\text{C}$ . All birds in each generation were sampled this way at the same time of day 6 weeks post-fledging. CORT concentrations were measured after extraction of 20  $\mu$ l aliquots of plasma in diethyl ether, by radioimmunoassay (Wingfield et al., 1992) using antiCORT antiserum (code B21-42 and B3-163, Esoterix Inc. Endocrinology, Calabasas Hills, CA, USA) and [ $^{1,2,6,7-3}\text{H}$ ]-CORT label (Amersham, Bucks, UK). The interassay coefficient of variation was 15.7%, and the intra-assay coefficient of variation was 3.1%. The mean extraction efficiency was 72%. The assay was run with 50% binding at 134 pg per tube, and the detection limit (for 7.3  $\mu$ l aliquots of extracted plasma) was 1.76 nmol  $\text{l}^{-1}$ .

Blood samples for testosterone assay were taken immediately upon capture, and the plasma obtained and stored in an identical manner to the CORT samples. The birds were sampled for testosterone 3 months post-fledging when they were fully adult. Testosterone concentrations were measured in plasma samples by direct radioimmunoassay using anti-testosterone antiserum (code 8680-6004, Biogenesis, Oxford, UK) and [ $^{125}\text{I}$ ]-testosterone label (code 07-189126, MPI Biomedicals Europe, Illkirch, France) (Parkinson and Follett, 1995). The assay was run with 50% binding at 11.0 pg per tube, and a detection limit of 0.068 nmol  $\text{l}^{-1}$  for the 20  $\mu$ l plasma volumes that were run in the assay. The interassay coefficient of variation was 15.5% and the intra-assay coefficient of variation was 2.2%.

#### *Immune challenges: general methodology*

Seventy-two adult birds taken randomly from the F5 generation were used to compare cell-mediated and humoral immune responses between CORT selection lines. The birds were divided equally between sex, CORT selection line and replicate selection line. To test whether either immune challenge changed the ability to respond to the other (i.e. whether a trade-off existed between different components of the immune system), half of the birds were first challenged by PHA (phytohemagglutinin) injection to test the cell-mediated

response and then challenged by diphtheria:tetanus injection to test their humoral response. The remaining birds were challenged in the opposite order, first with diphtheria:tetanus injection and then by PHA. The first group was injected with the first diphtheria:tetanus vaccine 6 days after the PHA injection, and the second group was injected with PHA 12 days after the second (final) diphtheria:tetanus injection. The timings of the challenges were based on previous work with these immune challenges (Martin et al., 2006; Svensson et al., 1998).

#### *Cell-mediated immune response*

Each individual was injected with the mitogen phytohemagglutinin (PHA; Sigma, St Louis, MO, USA) intradermally into the left wing web (Lochmiller et al., 1993). Each bird received 50  $\mu$ l of a suspension of 3 mg PHA-P in 1 ml phosphate buffered saline ( $1\times$ PBS). Similar concentrations of PHA solution have been used previously in similar experiments (e.g. Snoeijs et al., 2005). A spessimeter (Alpa s.r.l., Milan, Italy) was used to measure the wing web before injection (as a control measurement), and at 24 h after injection (to the nearest 0.01 mm), to measure the wing web swelling in response to the mitogen. At each occasion, the swelling was measured three times and the mean used in all further analyses. PHA has been found to elicit responses from both innate and acquired components of the immune system (see Martin et al., 2006).

#### *Humoral response*

The plasma collected at 6 weeks post-fledging was subsequently split between testing for CORT production and testing for naturally occurring antibodies to diphtheria:tetanus, as a control measurement before the injection of the vaccine. Each bird was immunised with 100  $\mu$ l of diphtheria:tetanus vaccine (Aventis Pasteur, Swiftwater, PA, USA) by intraperitoneal injection, an antigen challenge commonly used in studies of humoral immunocompetence in birds (e.g. Svensson et al., 1998; Ilmonen et al., 2000; Råberg et al., 2000; Owen-Ashley et al., 2004). In other studies of small passerine birds, the peaks of the primary and secondary response have occurred 12 and 8 days after antigen injection, respectively (Svensson et al., 1998; Hasselquist et al., 1999). Twelve days later blood samples were taken from the brachial vein (as described above). Twenty-one days after the initial injection the birds were inoculated again with diphtheria:tetanus vaccine to test the secondary humoral response. Eight days after this the birds were again blood sampled. In total three blood samples were taken from each bird. The amount of antibody present in each sample was determined by the use of ELISA [a full description of the methodology employed has been given previously (Hasselquist et al., 1999; Owen-Ashley et al., 2004)].

#### *Morphological measurements*

All the birds were weighed to the nearest 1 g using a Pesola spring balance, and their right tarsus lengths measured to the nearest 0.1 mm with digital callipers. To ensure accuracy, the mean of three measurements was taken for the tarsus length measurements. This procedure was highly repeatable ( $r=0.92$ ,  $F_{106,214}=36.89$ ,  $P<0.001$ ).

*Reflectance spectrophotometry*

A total of 48 males were randomly selected from each CORT line within the F4 generation. We used ultra-violet–human visible (UV–VIS) reflectance spectrophotometry to measure three regions considered by various workers (e.g. Burley et al., 1982; Burley and Coopersmith, 1987; Zann, 1996) to be sexually selected in the zebra finch: the leg (tarsus), the beak (upper mandible) and the cheek patch. Four reflectance spectra were taken from each region of each subject, with each leg and cheek patch of each subject measured twice. Each measurement was taken from a *ca* 2 mm diameter spot, at randomly chosen locations within each region. The spot was illuminated at 45° to the surface by a Zeiss CLX 500 xenon lamp (Zeiss, Jena, Thuringia, Germany) and reflected light collected at 135° (90° to illumination) using a Zeiss GK-21 goniometer. The spectra were measured using a Zeiss MCS 500 spectrophotometer, and illumination was always from the proximal/attachment end of the feather, bill or leg. Reflectance was calculated relative to a Spectralon™ (Labsphere, Congleton, Cheshire, UK) white standard, taken before starting every new region, with a dark current calibration before each measurement.

*Dominance trials*

A total of 36 males were taken at random from each CORT line and replicate from the F5 generation (the same males as used in the immunity experiments, but several months later). Each trial consisted of placing two males from the same replicate (1 or 2) but different CORT selection line (high, low or control) into small (50 cm × 50 cm × 100 cm) wooden cages fronted with wire mesh. An equal number of combinations of males from the different CORT lines were tested; therefore, nine trials of a total of 18 males from each replicate were carried out (3 trials of each combination from each replicate). The birds were provided with a water bottle and *ad libitum* food from a fountain feeder attached to the wire mesh. The males were housed in this way for 24 h pre-trial, to acclimatise them to their environment. Four hours before the trial the food hopper and any spilt food were removed. The trial commenced as soon as the food hopper was replaced; the positioning of the feeder (in the corner of the cage) resulted in only one bird being able to feed comfortably at any one time. The amount of time each previously food-deprived male spent at the hopper was recorded over a 20 min period, as were the number of aggressive displacements of one bird by another from the hopper. After this period the birds were generally satiated and no inference of rank could be made by feeding behaviour. The ‘winner’ of each trial was determined based upon the time spent at the feeder and the number of displacements carried out relative to the ‘losing’ male.

*Statistical analyses*

The degree of swelling exhibited by the wing web injected with PHA was used as the dependent variable in restricted maximum likelihood (ReML) models. This procedure assumes the residuals derived from the model are normal and homoscedastic but allows random terms to be fitted in addition to the fixed terms. The analyses were split between including testosterone titre in the model as an independent variate (when

only the males were included), and including ‘sex’ as a factor when both males and females were included. In both models, CORT selection line, body mass, tarsus length and peak CORT titre were included in the maximal model as fixed effects and replicate was included as the random term; a minimal model was derived by stepwise deletion of non-significant terms ( $P > 0.05$ ). The same procedure was followed for the models containing primary and secondary levels of anti-diphtheria and anti-tetanus antibodies as response variables.

Differences between the selection lines in body mass and tarsus length were analysed in ReMLs. For the F4 males, the fixed model consisted of selection line and CORT and testosterone titres for the tarsus comparisons; tarsus length was included in the fixed model for the body mass analyses. For the F5 birds, sex was included in the fixed model; otherwise, the model terms were identical to those used in the F4 model. Replicate was included as a random term in all the models.

Reflectance spectra were analysed using principal components analysis (PCA), which allows one to summarise the variation in reflectance into three components that explain almost all the variation: principal component 1 (PC1) describes achromatic brightness and accounts for most of the variation found, and PCs 2 and 3 represent chromatic variation (see Bennett et al., 1997; Cuthill et al., 1999; Cherry and Bennett, 2001; Cherry et al., 2007). The PC scores derived from the PCA were then included in ReMLs as the response variables. Significant results were interpreted according to the methodology employed in previous studies (Bennett et al., 1997; Cuthill et al., 1999; Cherry et al., 2007).

For the dominance trials, the number of seconds spent feeding and the number of aggressive displacements were the dependent variables in separate ReMLs; mass, tarsus length, CORT selection line, CORT titre and testosterone titre were the fixed terms, and trial and replicate line were the random terms. Finally, a generalized linear mixed model (GLMM) with a binomial distribution was used to determine which fixed effects (as above) affected the outcome of dominance trials (arbitrarily designated as ‘winner’ or ‘loser’ as judged by the time spent feeding and the number of aggressive displacements). In this case only replicate was included as the random term.

The residuals of all the above models were checked for homoscedasticity and normality, and where necessary the response variables were transformed appropriately. To ensure outliers did not unduly bias the significant results in certain models, we used the bootstrap procedure [terms are significant if their 95% confidence intervals (c.i.) do not straddle zero (Manly, 1997)]. To determine whether the large number of zero values obtained from the primary tetanus response in both sexes affected this analysis, in addition to running a ReML we also coded individuals as to whether they had responded or not to the immune challenge (1 or 0) and used a binomial model. Statistics shown in all tables were derived from maximal models that had been stepwise deleted to minimal models that included all fixed and random effects. All analyses were carried out using Genstat version 7 (Genstat 6th Edition, VSN International Ltd., Hemel Hempstead, Herts, UK) and S-Plus 2000 (Insightful Corporation, Seattle, WA, USA).

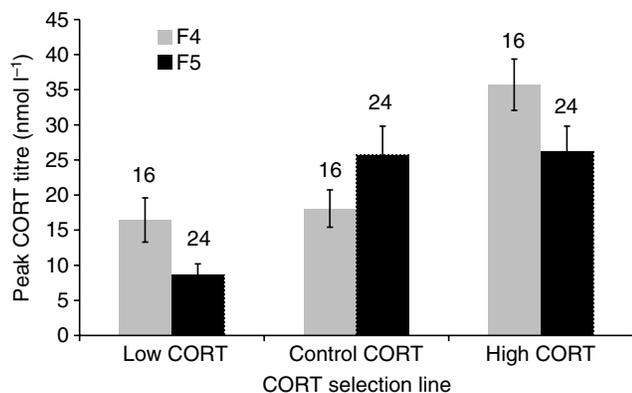


Fig. 1. Mean peak corticosterone (CORT) titres of zebra finches used in immunity and dominance experiments (F5) and in spectrophotometric comparisons (F4) according to selection line ( $\pm$  s.e.m.). Sample sizes are shown above the bars.

**Results**

*CORT titres*

The CORT titres of the birds used in the dominance trials and immunity experiments (from the F5 generation) differed significantly between selection line (Wald=39.76, d.f.=2,  $P < 0.001$ ; Fig. 1), as did the CORT titres of the birds (all males) used in the spectrophotometric study (from the F4 generation; Wald=96.99, d.f.=2,  $P < 0.001$ ; Fig. 1). There was a significant, negative relationship between CORT titre and testosterone titre in the F4 males ( $F_{1,43}=4.53$ ,  $P=0.039$ ; coefficient  $\pm$  s.e.m.:  $-0.072 \pm 0.034$ ), and a significant, positive relationship between the two hormones in the F5 males ( $F_{1,28}=5.52$ ,  $P=0.026$ ; coefficient  $\pm$  s.e.m.:  $0.037 \pm 0.016$ ).

*Cell-mediated immunity*

In an analysis including both males and females, there was no effect of sex (Wald=2.61, d.f.=1,  $P=0.106$ ), order of immune test (Wald=1.21, d.f.=1,  $P=0.271$ ) or CORT selection line (Wald=4.97, d.f.=2,  $P=0.083$ ) on PHA response, but there was a significant, positive relationship between CORT titre and PHA response (Wald=9.20, d.f.=1,  $P=0.002$ ; Fig. 2). There is a suggestion from Fig. 2 that this relationship was non-linear. We tested this by comparing the minimal model as a linear, quadratic or cubic regression. There was no significant difference between models (see Table 1), suggesting that the addition of extra parameters did not explain more variation than the linear model. Note, however, that there were few samples measured at high levels of CORT.

Testosterone titre had no relationship with the males' PHA response (Wald=1.79, d.f.=1,  $P=0.181$ ), and neither did

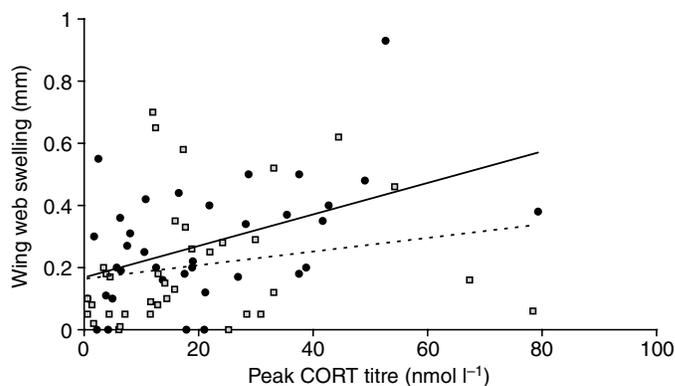


Fig. 2. Relationship between mean wing web swelling 24 h post injection of PHA and peak CORT titre. Grey squares with dashed trendline represent females; black circles and solid trendline represent males.

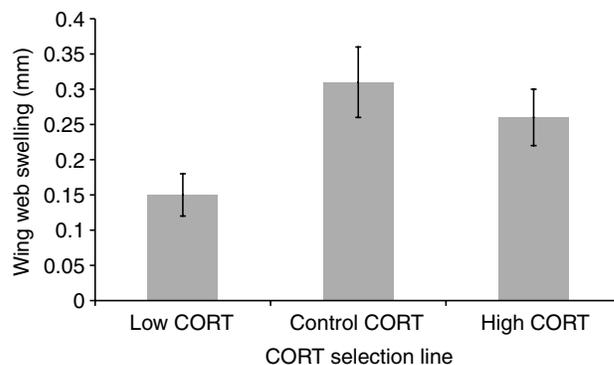


Fig. 3. Mean wing web swelling 24 h post injection of PHA of F5 males according to CORT selection line ( $\pm$  s.e.m.).

CORT selection line (Wald=2.15, d.f.=2,  $P=0.341$ ); again, CORT titre did have a significant, positive effect (Wald=7.39, d.f.=1,  $P=0.007$ ). When CORT titre was omitted from the model, CORT selection line had a significant effect (Wald=10.07, d.f.=2,  $P=0.007$ ; low CORT males had a lower response than males from the high and control CORT lines; see Fig. 3), suggesting that the two terms explained the same variation because CORT titres covaried with selection line (see Fig. 1).

*Humoral immunity*

In males and females, neither primary nor secondary anti-diphtheria antibody responses were significantly affected by CORT line (see Table 2), or order of immune test or any

Table 1. Comparison of regression models describing the relationship between CORT titre and PHA response in zebra finches

Model	Residual SS	d.f.	Model versus	F	P value
Linear	2.417	70	–	–	–
Quadratic	2.290	69	Linear	0.004	0.949
Cubic	2.216	68	Linear	0.003	0.997
Cubic	2.216	68	Quadratic	0.002	0.961

CORT, corticosterone; SS, sum of squares.

Table 2. The effect of CORT selection line on measures of humoral immunity in zebra finches

	Antibody response	Wald statistic	d.f.	P value
Diphtheria	Primary	1.33	2	0.514
	Secondary	0.28	2	0.868
Tetanus	Primary	0.37	2	0.830
	Secondary	7.18	2	0.028

covariate ( $P>0.05$ ). In addition, testosterone titre had no significant relationship with the primary or secondary diphtheria response in the males ( $P>0.05$ ). However, there was a non-significant negative relationship between testosterone and the secondary anti-diphtheria antibody response in the males (Wald=3.34, d.f.=1,  $P=0.067$ ). There were no significant differences in any humoral immune response between the sexes ( $P>0.05$ ).

Testosterone did not have any relationship with the primary anti-tetanus antibody response, but did have a significant, negative relationship with the secondary tetanus response (Wald=14.46, d.f.=1,  $P<0.001$ ; testosterone titre 95% c.i. values: -10.98, -5.12). Order of immune test had an effect on secondary tetanus response within the males; the group that received the PHA challenge before the humoral challenge had a significantly lower secondary tetanus antibody response than the males that were not first challenged by PHA injection (Wald=9.49, d.f.=1,  $P=0.002$ ; order of immune challenge 95% c.i. values: 1.69, 5.15). When combining the two sexes, CORT titre had a significant, negative effect on the primary tetanus antibody response (Wald=16.38, d.f.=1,  $P<0.001$ ; Fig. 4; 95% c.i. values: -0.10, -0.03).

The binomial model revealed that birds with higher CORT titres were significantly less likely to respond to a primary tetanus challenge (Wald=11.7, d.f.=1,  $P<0.001$ ). In addition, we repeated the original analysis omitting the zero values. The effect of CORT titre fell out of the model in this analysis (Wald=0.02, d.f.=1,  $P=0.880$ ); however, there was a significant effect of CORT selection line on the secondary tetanus antibody response (Wald=7.18, d.f.=2,  $P=0.028$ ; control birds exhibited a lower response than birds in the high and low CORT lines; see Fig. 5), and this response was also significantly and positively affected by body mass (Wald=7.34, d.f.=1,  $P=0.007$ ). The minimal model was bootstrapped and this confirmed the significance of body mass and CORT line (mass 95% c.i. values: 0.44, 1.74; CORT line 95% c.i. values: -4.19, -0.12). See Table 2 for a full description of the statistical values derived from the analyses for CORT selection line.

#### Reflectance spectrophotometry

See Table 3 for a summary of the results for CORT selection line, including the non-significant results.

There was a significant effect of CORT line on leg PC3 score (Wald=9.21, d.f.=2,  $P=0.01$ ; Fig. 6). The low CORT line had the peak values (Fig. 6). Leg PC3 was a chromatic variable with highest loadings in the UV and lowest loadings in the blue wavebands (Fig. 7).

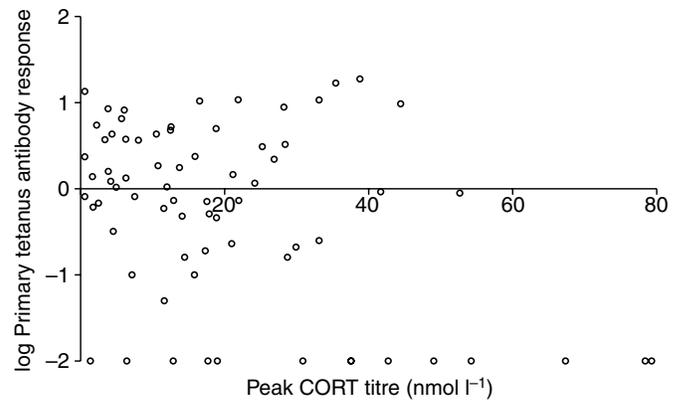
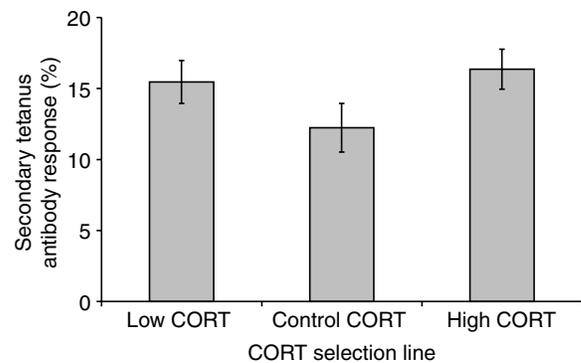
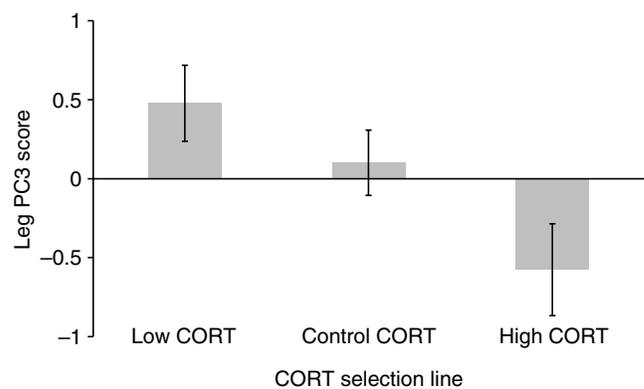


Fig. 4. Relationship between peak CORT titre and primary tetanus antibody response.

Fig. 5. Mean secondary tetanus antibody response according to CORT selection line ( $\pm$  s.e.m.).Fig. 6. Mean leg PC3 scores according to CORT selection line ( $\pm$  s.e.m.).

There was also a significant effect of CORT line on cheek PC2 score (Wald=11.58, d.f.=2,  $P=0.003$ ; Figs 8, 9). The control line had higher positive PC scores than the other lines (Figs 8, 9). Cheek PC2 was a chromatic variable and had lowest loadings in the long wavelengths and highest loading in the UV-blue wavebands (Fig. 9). No effect of CORT selection line was found for any beak PC score or leg PC1 and cheek PC1 and 3 scores (see Table 3). There was no significant relationship with

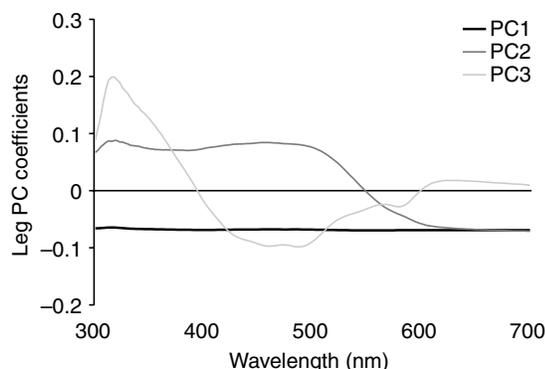


Fig. 7. Coefficients of the first three principal components (PCs) of the leg region plotted against wavelength.

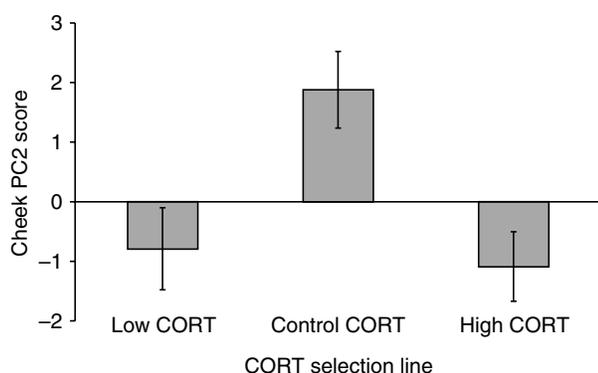


Fig. 8. Mean cheek PC2 scores according to CORT selection line ( $\pm$  s.e.m.).

testosterone or CORT titre for any of the regions measured ( $P>0.05$  for all tests).

#### Dominance trials

There was no significant difference in the amount of time spent at the feeder between males from the different CORT selection lines (Wald=2.09, d.f.=2,  $P=0.351$ ). Both tarsus length (Wald=4.73, d.f.=1,  $P=0.030$ ) and body mass (Wald=4.79, d.f.=1,  $P=0.029$ ) had significant effects on time spent feeding (coefficients  $\pm$  s.e.m., tarsus:  $9.2\pm 4.2$ ; mass:  $-2.3\pm 1.1$ ). CORT selection line had no effect on the number of aggressive displacements (Wald=0.14, d.f.=2,  $P=0.933$ ). The number of trials 'won' was not significantly affected by any of the terms in the model (CORT line: Wald=0.31, d.f.=2,  $P=0.856$ ; all other terms  $P>0.05$ ).

#### Size and mass

The F4 males in the low CORT lines were significantly larger in terms of tarsus length than males in the high CORT lines (Wald=8.84, d.f.=2,  $P=0.012$ ; see Fig. 10). There was no significant difference in mass after correcting for tarsus length (Wald=0.25, d.f.=2,  $P=0.884$ ). Individuals in the low corticosterone lines in the F5 generation were again significantly larger than those in the high corticosterone lines (Wald=6.54, d.f.=2,  $P=0.038$ ; Fig. 10). When the analysis was split by sex there was no significant difference between CORT

Table 3. The effect of CORT selection line on reflectance from sexually selected traits in male zebra finches

Region	PC score	Wald statistic	d.f.	P value
Beak	1	2.54	2	0.280
	2	3.30	2	0.192
	3	0.07	2	0.968
Leg	1	0.85	2	0.654
	2	3.83	2	0.148
	3	9.21	2	0.010
Cheek	1	0.38	2	0.826
	2	11.58	2	0.003
	3	2.66	2	0.265

PC, principal component.

lines in tarsus length within each sex (males: Wald=3.41, d.f.=2,  $P=0.182$ ; females: Wald=4.21, d.f.=2,  $P=0.122$ ). We conducted a power analysis to test whether the non-significant difference in tarsus length in the males was due to small sample size. This confirmed that 62 males would have been required to detect a significant difference. Overall, there was no difference in mass (Wald=1.68, d.f.=2,  $P=0.433$ ) after correcting for tarsus length. There was a significant effect of generation on tarsus length, such that F5 individuals were larger than the F4 birds (Wald=21.68, d.f.=1,  $P<0.001$ ; Fig. 10). Neither CORT nor testosterone titre had any significant effect on size or mass, nor were there any significant sex differences; however, there was a non-significant trend for females to be heavier than males (mass: Wald=3.58, d.f.=1,  $P=0.059$ ) and males to be larger than females (tarsus length: Wald=2.94, d.f.=1,  $P=0.086$ ).

## Discussion

### Immunity

The results from these experiments suggest that selection for increased peak CORT production may be associated with immunosuppressive effects on the humoral response, but CORT appears to be immuno-enhancing in relation to the cell-mediated response. It is entirely possible, however, that the hormone is not directly responsible, but that pleiotropic effects have occurred during selection such that selection for increased peak CORT level has selected for a reduction in humoral immunocompetence and an increase in cell-mediated immunocompetence. We cannot separate these two possibilities. However, regardless of whether the effect is direct or indirect it shows a link between the mechanism promoting peak CORT production and immune function. Although testosterone had no effect on the cell-mediated response, it exhibited a significant negative relationship with the secondary antibody response to tetanus injection, and a close to significant negative relationship with the secondary anti-diphtheria antibody response. In addition, we have evidence to suggest that the two arms of the immune system do trade-off, but only in males.

Not only does there appear to be a trade-off between the cell-mediated response and the humoral immune function, but also the two measures of immunity are affected differentially by testosterone and CORT within the same individuals. CORT titre was positively related to the cell-mediated response,

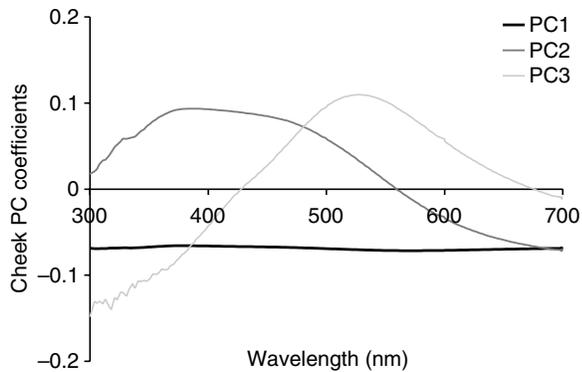


Fig. 9. Coefficients of the first three PCs of the cheek region plotted against wavelength.

whereas testosterone showed no significant relationship. Conversely, both hormones showed a negative relationship with antibody response. The primary antibody response to tetanus challenge was dependent upon CORT titre; birds with the highest levels of the steroid did not respond at all, whereas CORT levels did not affect those birds that did respond. Although there were significant relationships between CORT and testosterone titres and antibody response, there was little indication of a systematic difference between the CORT selection lines in immune response. The only difference found between the lines in humoral immunity was in response to the second tetanus injection; the control lines exhibited a lower antibody response than the high and low CORT lines. This result is difficult to interpret in terms of CORT titre, or indeed in terms of the selection for divergent levels of CORT, because it was the control line that differed from the two lines under directional selection. We suggest that this may be an artefact of the selection process, and the control lines exhibit an idiosyncratically reduced secondary response to tetanus antigen. It is possible that we not only selected for peak plasma CORT levels but also incidentally selected for genes involved in immune function; this may explain the results we obtained. However, there have been no pleiotropic effects of the selection programme on other variables, such as testosterone, or on several life history traits (see Evans et al., 2006). Another important point to note is that the birds used in this study were selected on peak levels of CORT. We have no evidence to suggest that they differed significantly in baseline CORT or that the two CORT measurements covaried. Therefore, we cannot rule out the possibility that baseline CORT levels influenced immune response in a different manner to peak levels.

Previous lab-based experiments on small mammals have generally found both CORT and testosterone to be immunosuppressive (see Harvey et al., 1984; Grossman, 1985). There is, however, less evidence in birds that either hormone has a negative effect on immune function [see Roberts et al. (Roberts et al., 2004) for a review of testosterone]; indeed in reptiles CORT has been found to have an immuno-enhancing effect (Svensson et al., 2002). The results from our experiments suggest that peak CORT is immuno-enhancing in the case of the cell-mediated response, but is immunosuppressive in the

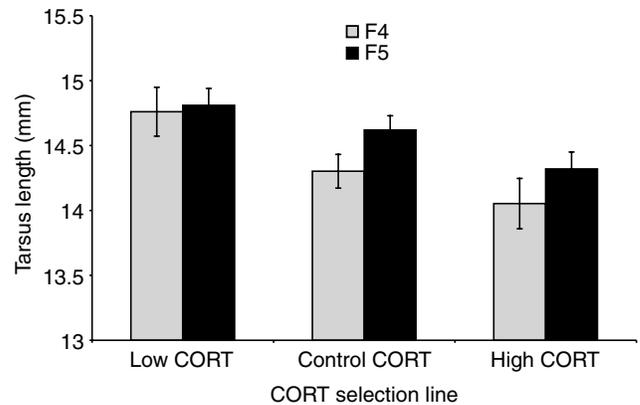


Fig. 10. Mean tarsus length of zebra finches from the F4 and F5 generations of selection according to selection line ( $\pm$  s.e.m.).

case of the antibody response. These results agree with studies in mammals that have found that high peak levels of glucocorticoids enhance cell-mediated immunity (Dhabhar and McEwen, 1999; Dhabhar, 2000). This may be because in stressful situations (and therefore when individuals are exhibiting high peak CORT levels) certain parts of the immune system are activated (for a review, see Dhabhar, 2002). Testosterone appears to have no effect on cell-mediated immunity but is negatively related to the antibody response. It is important to note that PHA injection a few days prior to diphtheria:tetanus injection significantly reduced the birds' ability to raise an antibody response. This trade-off has been found previously (e.g. Buchanan et al., 2003), and needs to be taken into account in future studies. What is particularly interesting is the fact that this trade-off was only seen in the males, suggesting sex differences in immune function that could be related to testosterone. There was, however, no reciprocal cost of mounting an antibody response on the ability to respond to PHA injection. Because testosterone levels were not manipulated, and there was little effect of CORT selection line on immune response, it should be noted that these results are correlational and do not necessarily suggest cause and effect; nevertheless, significant relationships (both positive and negative) existed between CORT and testosterone and immune response. In addition, when the analysis was carried out leaving CORT titre out of the model, CORT selection line did have a significant effect on PHA response.

In a previous manipulative study (testosterone implantation) on birds from these lines, it was found that high levels of CORT and testosterone in the same individuals were related to an enhanced antibody response (Roberts et al., 2007b). In addition, differences were found between the lines in the relationship between testosterone and antibody response, in that testosterone was found to be immunosuppressive but only in the high CORT line and when plasma levels of CORT were low (Roberts et al., 2007b). Although the results from this correlational study do not replicate the results from the previous manipulative study, both studies found no consistent immunosuppressive effect of peak corticosterone across different immune challenges – indeed both studies found that production of peak CORT was immuno-enhancing under

certain circumstances. Most significantly, selection for high levels of peak CORT resulted in no deleterious effect on immune function in either study.

Body mass had a significant, positive relationship with the secondary antibody response to tetanus. This suggests that the birds with the greatest antibody response were those with the largest initial fat reserves (as skeletal body size was corrected for in the analyses). Raising an antibody response may be costly (Deerenberg et al., 1997; Råberg et al., 2000; Hanssen et al., 2004) and could be related to general body condition (Ots et al., 2001); the positive relationship between initial body mass and antibody response supports this supposition.

#### *Morphometric parameters*

In both generations, the birds selected for low CORT stress responses were skeletally larger than the high CORT individuals. This result may suggest that overall the high CORT birds did have higher baseline levels of the hormone (although we have no evidence to confirm this), and chronic elevation of CORT resulted in possible developmental stress and consequently a smaller body size. Previous studies in zebra finches have found that increasing stress during ontogeny by artificially increasing brood size negatively affects offspring skeletal growth, and this effect is carried over between generations (Naguib and Gil, 2005; Naguib et al., 2006). In addition, Spencer et al. (Spencer et al., 2003) found that CORT administration to zebra finch nestlings significantly reduced their growth rates. Previous experiments in which peak CORT has been divergently selected for in birds have also found a negative effect of high CORT on development, but in relation to fluctuating asymmetry rather than absolute size differences (see Satterlee et al., 2000).

Our results support the hypothesis that developmental stress (or more correctly high CORT levels during ontogeny) adversely affects skeletal growth and subsequent adult size. This effect may well have significant implications for both mate choice and male–male competition.

#### *Reflectance spectrophotometry*

The analyses of reflectance spectra suggest that the effects of CORT on males' legs were chromatic and concentrated in the UV and blue regions of the spectrum. The legs of the low CORT and high CORT males differed significantly in PC3. The low CORT males' legs reflected more in the UV relative to the blue waveband compared with the high CORT males' legs. Female zebra finches are thought to prefer males with red legs (Burley et al., 1982; Zann, 1996), but no significant differences were found between the lines in leg redness *per se*. Male cheek patch colouration is also thought to be important in female choice in zebra finches (Immelmann, 1959), although it is unclear what characteristics of the cheek patch females prefer. The males from the control lines possessed cheek patches that were more reflective in the UV and blue wavelengths relative to longer (redder) wavelengths than the other lines. CORT titres (according to selection line) themselves played no part in spectrophotometric differences in cheek patch colouration, therefore the differences may be attributable to pleiotropic selection for these traits in the lines; or it is possible that inbreeding depression has had a differential effect on the sexual

signals of males from the high and low CORT lines and males from the control lines (the control lines were selected randomly each generation, whereas there existed a greater probability of siblings being selected in the other lines).

#### *Dominance*

Selection for different levels of peak CORT did not have any effect on dominance or aggression, and neither did testosterone. Perhaps not surprisingly, skeletal size was the main predictor of time spent feeding during the dominance trials. This may have been due to either large size resulting in physical superiority or a greater requirement for dietary resources, or it may have been a combination of the two factors. The fact that CORT had no effect on dominance ranking does not lend support to hypotheses that contend that CORT influences rank (see Creel, 2001); the results of our experiments agree rather with previous studies that have found no effect of CORT on dominance (Schoeche et al., 1997; Parker et al., 2002). However, the effect that peak CORT may have on dominance ranking may depend on environmental conditions, and consequently the stress experienced by the individual birds (Rohwer and Wingfield, 1981). Additionally, peak CORT may be less relevant than basal CORT in influencing dominance behaviour between males. Given that significant differences were found between the lines in tarsus length, it seems somewhat surprising that CORT line did not predict dominance behaviour whereas tarsus length did. However, in the F5 birds there was no significant difference between the males in tarsus length, possibly due to a smaller number of males measured than in the F4 generation resulting in the loss of power. Despite the F5 low CORT males tending to be larger than the F5 high CORT males this was insufficient to explain differences in dominance between them, and dominance could only be directly linked with actual skeletal size regardless of selection line.

#### *Conclusion*

Our selection experiment showed that peak CORT titre exhibits a positive relationship with the cell-mediated immune response, but at high levels may have a suppressive effect on the antibody response in zebra finches. The cell-mediated response appears to be costly, and a trade-off exists between the two measures of immunity. Peak CORT appears to have little direct effect on the reflectance characteristics of several sexually selected regions of male zebra finches, and has no effect on dominance ranking between males. Birds selected for low peak CORT level were skeletally larger than birds selected for high levels of CORT. From the results of our experiments, heritably high and low levels of stress, measured as peak CORT titre, have no effect on dominance rank or the quality of male sexual signals in the zebra finch. Although selection for divergent levels of CORT did not result in a significant difference in immunity between lines, actual CORT titres had a significant, positive effect on cell-mediated immunity but a negative effect on a measure of humoral immunity. Overall, our results provide no evidence that peak CORT plays a role in male dominance hierarchies or sexual signal quality; nevertheless, high levels do adversely affect skeletal growth, which may well have significant consequences on a plethora of life history traits in free-living birds.

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