

Juveniles exposed to embryonic corticosterone have enhanced flight performance

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Exposure to maternally derived glucocorticoids during embryonic development impacts offspring phenotype. Although many of these effects appear to be transiently ‘negative’, embryonic exposure to maternally derived stress hormones is hypothesized to induce preparative responses that increase survival prospects for offspring in low-quality environments; however, little is known about how maternal stress influences longer-term survival-related performance traits in free-living individuals. Using an experimental elevation of yolk corticosterone (embryonic signal of low maternal quality), we examined potential impacts of embryonic exposure to maternally derived stress on flight performance, wing loading, muscle morphology and muscle physiology in juvenile European starlings (*Sturnus vulgaris*). Here we report that fledglings exposed to experimentally increased corticosterone *in ovo* performed better during flight performance trials than control fledglings. Consistent with differences in performance, individuals exposed to elevated embryonic corticosterone fledged with lower wing loading and had heavier and more functionally mature flight muscles compared with control fledglings. Our results indicate that the positive effects on a survival-related trait in response to embryonic exposure to maternally derived stress hormones may balance some of the associated negative developmental costs that have recently been reported. Moreover, if embryonic experience is a good predictor of the quality or risk of future environments, a preparative phenotype associated with exposure to apparently negative stimuli during development may be adaptive.

Keywords: yolk hormones; corticosterone; embryonic stress; flight performance; survival; European starling

1. INTRODUCTION

Maternally derived stress hormones (glucocorticoids) significantly impact offspring phenotype across phylogenetically diverse taxa (McCormick 1998; Seckl 2004; Uller & Olsson 2006; Love & Williams 2008a). Indeed, a mother’s quality or the quality of the environment she reproduces in can act as a maternal effect via embryonic hormonal mechanisms (Gluckman *et al.* 2005; Love & Williams 2008a,b). Embryos and fetuses can be exposed to maternal stress hormones via the placenta in mammals (see Seckl 2004) and via their presence in eggs of oviparous species (Love *et al.* 2005; Saino *et al.* 2005; Hayward *et al.* 2006; Lovern & Adams 2008). Embryonic exposure to maternal glucocorticoids is known to cause both short-term ‘transient’ and preparative ‘programming’ effects in mammals and birds (Seckl 2004; Gluckman *et al.* 2005; Love & Williams 2008b). Immediate effects in free-living species can include reductions in hatch or birth mass/structural size, reduced growth, compromised immunity and even reduced survival (Rubolini *et al.* 2005; Saino *et al.* 2005; Love & Williams 2008a), whereas long-term effects can include programming of key behavioural and physiological pathways (Uller & Olsson 2006; Love & Williams 2008b).

Studies have recently begun to examine offspring phenotypic responses to maternally derived yolk hormones within an evolutionary framework (see Groothuis *et al.* 2005; Love *et al.* 2005), and we are beginning to understand the influence of these maternal effects on fitness (Groothuis *et al.* 2005; Marshall & Uller 2007; Love & Williams 2008a). However, it is not directly obvious how many of the phenotypic responses studied thus far (changes in body mass/size, growth and immunity) affect fitness. Examining a trait that is known to directly impact survival would significantly increase our understanding of the relative evolutionary and ecological importance of these maternal hormonal effects. In birds, flight performance, including take-off velocity and angle of trajectory, is a phenotypic trait with likely fitness consequences (Lima 1993; Witter *et al.* 1994; Lee *et al.* 1996). Survival can be influenced by initial take-off, as predator captures are reduced when prey are fully airborne (Cresswell 1993). As post-fledging predation-induced mortality can be high (Naef-Daenzer *et al.* 2001), flight performance should be a heavily selected trait during this life-history stage. Although post-natal exposure to developmental stress is known to affect muscle mass in juveniles (Lin *et al.* 2007) and adults (Gray *et al.* 1990), with potential effects on muscle metabolism (Lin *et al.* 2007), we know little to nothing of the effects of embryonic stress on these performance-related systems.

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Here we use an experimental manipulation of the quality of the embryonic developmental environment to examine how maternally derived yolk corticosterone affects future flight performance in a free-living passerine, the European starling (*Sturnus vulgaris*). We use yolk corticosterone injections to manipulate embryonic exposure to maternal stress (Love *et al.* 2005, 2008; Love & Williams 2008a,b), a manipulation that is biologically relevant since mothers in poor condition (Love *et al.* 2005) and those that face increased predation risk (Saino *et al.* 2005) appear to deposit more corticosterone into eggs. Recently, researchers have suggested that embryonic exposure to maternal stress may provide offspring with a signal of the quality of their future environment (Love *et al.* 2005; Love & Williams 2008a) and may even adaptively programme particular physiological and behavioural pathways (Gluckman *et al.* 2005; Love & Williams 2008b; Mathis *et al.* 2008). Embryonic exposure to elevated maternally derived stress hormones may therefore have the potential to match offspring flight performance to the quality (i.e. risk of predation) of their post-natal environment. If so, we would predict that embryonic exposure to elevated maternally derived corticosterone would increase future flight performance in juveniles.

2. MATERIAL AND METHODS

(a) Manipulation of embryonic stress

Research was conducted from April to July 2005 at Davistead Dairy Farms in Langley, British Columbia, Canada under a Simon Fraser University Animal Care permit (657B-96) following guidelines of the Canadian Council on Animal Care. Nest-boxes were checked daily to determine clutch initiation, laying sequence and clutch completion dates. Starlings at the field site lay 5.9 ± 0.2 (mean \pm s.e.) eggs per clutch within the main peak of laying, incubate for 10.3 ± 0.1 days and fledge nestlings 22 ± 0.9 days following hatching (Love *et al.* 2005). Yolk corticosterone levels were manipulated as per Love & Williams (2008a). Briefly, nests were randomly divided into oil injected ($n=32$) and corticosterone injected ($n=32$); an additional group of unmanipulated eggs ($n=9$) were also included (see 'statistical analysis' section). Fresh eggs were injected within 3 hours of laying and the corticosterone manipulation elevated mean yolk corticosterone concentrations by 1.5 s.d. from the population mean (from 15.4 to 28.3 ng g⁻¹ based on Love *et al.* 2008). Just prior to hatching, eggs were exchanged with wooden eggs and placed in an incubator until hatching. Hatchlings were returned to their nest and nestling identity and age were tracked using non-toxic food colouring and nestling-specific feather trimming; at 10 days of age, chicks were banded to allow for individual identification. All nestlings were weighed and measured (exposed culmen, metatarsus, wing) at the ages of 0, 5, 10, 15, 17 and 21 days; flattened wing cord measurements began at 10 days when primary feathers began to appear.

(b) Flight performance trials and characterization of performance traits

Fledglings were collected from their nest-box on the morning of the brood mean 21st day of age and placed in cloth bags until the flight performance trials. The time in bags did not exceed 60 min (groups not different in holding times: t -test,

$t=0.19$, $p=0.42$) and this short holding period is not sufficient for transcription and translation of the subsequently measured muscle enzymes. The performance chamber consisted of a wooden frame $2.5 \times 1 \times 1$ m covered with fine plastic netting (top and left end), black plastic sheeting (bottom), clear Plexiglas (front) and a white plastic back with a 10×10 cm grid. Given the important difference between alarmed and non-alarmed (also called routine or spontaneous) flight (Kullberg *et al.* 1996; Kullberg 1998; Veasey *et al.* 1998; Nudds & Bryant 2002), we initially modelled our chamber on that of Kullberg *et al.* (2002a,b), with the bird being released at the bottom of the vertical chamber and vertical take-off ability being measured during the simulated escape flight. However, while this method was effective for adult birds, fledglings proved incapable of sustained vertical take-off. As such, the chamber was rotated to a horizontal position. To ensure that take-off was independent of any handling effects at the time of release, birds were introduced into the chamber via a short (approx. 30 cm long) tube, 10 cm in diameter and aligned at a 30° incline designed to simulate the first flight from the nest-box. Upon release, over 90 per cent of fledglings would launch into flight down the chamber (after emerging from the 'nest-hole') and would fly straight into the mesh at the end of the chamber (as though it was unseen).

Flight was videotaped with a digital-8 video camera (Sony TRV720; 30 frames s⁻¹) placed 2 m from the front of the chamber. To prevent habituation to the process, fledglings were subjected to only two flight trials. After flight trials, structural measurements (beak length, tarsus length, tail feather length and flattened wing chord) and body mass (to 0.01 g) were collected. A digital photograph of the left wing (standardized in a bent wing position against a scaled board) was taken to calculate wing area (using the total pixel method in PHOTOSHOP v. 7.0, Adobe Systems Inc.), which was used to calculate wing loading, that is, the ratio of an individual's body weight to wing area (Pennycuik 1972). Birds were then collected (University of British Columbia Animal Care Permit, no. A04-0277), a subsample of the left pectoralis major muscle was immediately collected and frozen in liquid nitrogen for enzymatic analyses, and the carcasses were immediately refrigerated. Within 2–3 hours, both the pectoralis major and supracoracoideus muscles, which together comprise the principal flight musculature, were removed from the right side of the bird's body via dissection. Fresh muscle mass was recorded to 0.0001 g and the tissue stored at -20°C. Birds were visually sexed based on the presence of testes or an ovary (confirmed via PCR sexing techniques following Love *et al.* 2005). Muscles were freeze-dried (Virtis Freezemobile model 8ES) and fat-extracted using a Soxhlet apparatus with petroleum ether to determine lean dry pectoral muscle (LDPM) mass.

Flight trials were analysed on a TV monitor. Vertical displacements (to the nearest 2.5 cm) and associated times (to the nearest quarter frame) were measured relative to the 10 cm grid at 0.5 m horizontal intervals, with the centre of the head used as a reference. Flight time taken per interval was thus calculated as the number of frames $\times 1/30$ s frame⁻¹. As the 0 m mark was set 0.21 m forward from where the bird emerged, analysis began after approximately one wing beat and once the bird's feet had left the perch. These parameters allowed the calculation of an average mechanical energy per unit mass E (J kg⁻¹) for each interval according to the

equation from Williams & Swaddle (2003),

$$E = \frac{1}{2}(V_x^2 + V_z^2) + gz,$$

where V_x and V_z are the horizontal and vertical components of flight velocity, respectively; g is the acceleration due to gravity; and z is the height (Williams & Swaddle 2003). This measure was chosen since it describes both the height and velocity gain components of flight performance in a single variable (Williams & Swaddle 2003). Therefore, the energy gain between the first interval (0–0.5 m) and the third interval (1–1.5 m) was determined and used for the purpose of our study as a measure of overall flight performance (referred to hereafter as ‘flight performance’). In all cases, the best performance from the two trials was taken.

(c) Enzymatic assays

To better understand the enzymatic determinants of flight performance, we measured the maximum catalytic activity of key enzymes from various metabolic pathways. Assays were performed at 40°C using a Spectra Max Plus 384, 96-well microplate reader (Molecular Devices, Sunnyvale, CA, USA), and were adapted from Burness *et al.* (2005). Briefly, frozen, powdered muscle samples were homogenized on ice in 19 volumes of homogenization buffer (20 mM Hepes, 1 mM EDTA, 0.1% Triton-X 100, pH 7.0). 3-hydroxyacyl CoA dehydrogenase (HOAD) and lactate dehydrogenase (LDH) were assayed in 50 mM Imidazole (pH 7.0) at 340 nm; pyruvate kinase (PK) and creatine phosphokinase (CPK) were assayed at pH 7.4. Citrate synthase (CS) was assayed in 20 mM Tris (pH 8.0), 412 nm. Enzyme activities are expressed as micromoles substrate converted to product per minute per gram of tissue (U g^{-1}). Assay conditions were as follows: CS: 0.1 mM 5,5′ dithiobis (2-nitrobenzoic acid, DTNB), 0.15 mM acetyl CoA and 0.5 mM oxaloacetate (omitted from control). LDH: 0.15 mM NADH, 1 mM pyruvate. PK: 0.15 mM NADH, 5 mM ADP, 100 mM KCl, 10 mM MgCl_2 , 10 μM fructose 1,6 bisphosphate, 5 mM phospho(enol)pyruvate, excess LDH. 3-HOAD: 0.15 mM NADH, 0.1% Triton X-100, 0.1 mM acetoacetyl CoA (omitted from the control). CPK: 0.5 mM NAD, 1 mM ADP, 5 mM glucose, 10 mM AMP, 5 mM MgCl_2 , 50 mM phosphocreatine (PCr), excess hexokinase and glucose-6-phosphate dehydrogenase. Protein content was determined using a Bradford Assay (Bio-Rad Inc., Hercules, CA, USA).

(d) Statistical analysis

We used general linear mixed models to examine the effects of experimental treatment on (i) flight performance and (ii) traits predicted to influence flight performance. Traits considered were body mass, LDPM and wing loading, and factors related to muscle quality were water content, fat content and metabolic enzyme activity. Treatment and sex were included as fixed factors and all models were fitted using the restricted maximum-likelihood procedure in JMP v. 6.0 (SAS Inc.), with ‘maternal identity’ specified as a random factor nested within treatment to control for the non-independence of siblings in the analysis. There was no significant difference at fledging between individuals hatching from oil-injected eggs and unmanipulated eggs for any of our physiological, morphological or performance parameters (each $p > 0.05$; O.P. Love & T.D. Williams 2004, unpublished data); groups were therefore pooled together and considered together as control eggs.

3. RESULTS

There were no differences between treatments for the following traits at hatching: hatching success, hatching brood sizes and hatching brood sex ratios (Love & Williams 2008a). There were also no differences for parental feeding rates during development, nor for the following traits at fledging: fledging success, body mass and structural size (Love & Williams 2008a).

Fledglings from corticosterone-treated eggs performed better during flight performance trials than did chicks from control eggs (treatment: $F_{1,34} = 3.96$, $p = 0.05$; sex \times treatment: $F_{1,50} = 0.06$, $p = 0.81$; figure 1a). Corticosterone-exposed fledglings had significantly heavier lean dry pectoral muscles at fledging than control fledglings controlling for body mass (treatment: $F_{1,34} = 10.65$, $p = 0.0025$; sex \times treatment: $F_{1,56} = 0.20$, $p = 0.65$; figure 1b), with females having heavier lean dry pectoral muscles than males ($F_{1,56} = 4.08$, $p = 0.04$, controlling for body mass). The muscles of corticosterone-injected nestlings had a lower water content at fledging than control fledglings (treatment: $F_{1,34} = 15.44$, $p < 0.001$; sex \times treatment: $F_{1,57} = 0.007$, $p = 0.93$; figure 1c); that is, they had greater functional maturity. Corticosterone-exposed fledglings had a lower wing-loading than did control nestlings (treatment: $F_{1,34} = 4.89$, $p = 0.033$; sex \times treatment: $F_{1,58} = 0.32$, $p = 0.58$; figure 1d) due to a larger wing surface area (treatment: $F_{1,34} = 7.65$, $p = 0.009$; sex \times treatment: $F_{1,58} = 0.11$, $p = 0.74$). However, there was no treatment difference in fat content (treatment: $F_{1,34} = 1.86$, $p = 0.18$; sex \times treatment: $F_{1,57} = 1.08$, $p = 0.30$).

Corticosterone-exposed fledglings had higher citrate synthase (CS) activity (treatment: $F_{1,21} = 4.90$, $p = 0.04$; sex \times treatment: $F_{1,21} = 0.20$, $p = 0.66$; figure 2a), and marginally higher creatine phosphokinase (CPK) activity (treatment: $F_{1,21} = 3.86$, $p = 0.06$; sex \times treatment: $F_{1,21} = 0.01$, $p = 0.92$; figure 2b) than control chicks. No other enzymes differed with treatment (all $p > 0.10$). However, when CS and CPK activity were corrected for muscle protein content, there was no longer a significant effect of treatment (each $p > 0.20$). Thus, differences in enzyme activity appear largely to reflect differences in the functional maturity of the muscle.

4. DISCUSSION

(a) Stress-induced flexibility in offspring phenotype

We identified a positive effect of elevated embryonic exposure to maternal stress on offspring phenotype, namely an increase in flight (escape) performance at fledging. Mechanistically, our results indicate that the increased performance was probably due to a combination of (i) an acceleration of muscle development during the nestling phase (resulting in an increase in pectoral muscle mass and higher muscle metabolic capacity at fledging) and (ii) increased wing area (resulting in decreased wing loading). Interestingly, however, flight performance and the mechanical machinery underlying flight was not affected by *in ovo* corticosterone exposure in the same sex-specific manner as recently reported for other traits, i.e. size at hatching, growth, immune function and survival (Love *et al.* 2005; Hayward *et al.* 2006; Satterlee *et al.* 2007; Love & Williams 2008a). These results appear to indicate therefore that embryonic exposure to maternal stress has

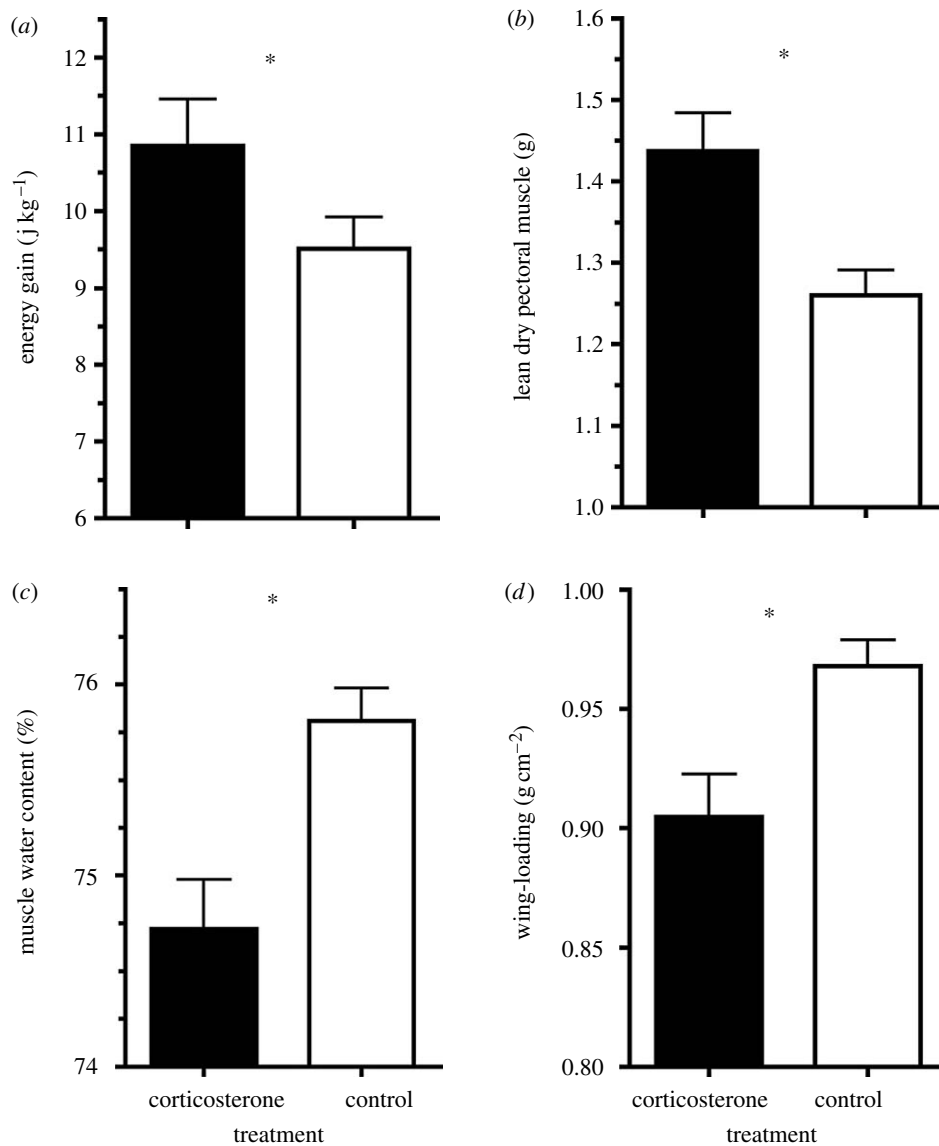


Figure 1. Effects of embryonic exposure to elevated yolk corticosterone on (a) flight performance, (b) LDPM mass, (c) pectoral muscle water content and (d) wing loading of fledgling European starlings. Values are mean \pm s.e.m.; asterisk denotes $p \leq 0.05$.

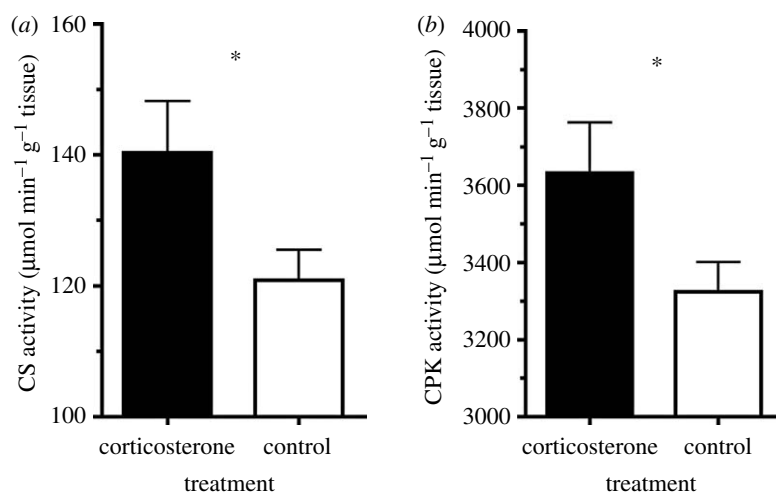


Figure 2. Effects of embryonic exposure to elevated yolk corticosterone on (a) CS activity and (b) CPK activity in the pectoral muscle of fledgling European starlings. Values are mean \pm s.e.m.; asterisk denotes $p \leq 0.05$ for CS and $p \leq 0.06$ for CPK.

sex-independent preparative effects on flight ability at fledging, i.e. preparing for maximal flight performance in response to a signal about the quality of the post-natal environment is equally important for both sexes in starlings.

(b) Mechanisms behind stress-induced flexibility in flight performance

Pectoral muscle mass and wing area are the two best predictors of flight performance (Marden 1987; Verspoor

et al. 2007). Consistent with this observation, fledglings exposed to elevated corticosterone *in ovo* had heavier and more functionally mature pectoral muscles (i.e. lower % water content; see [Ricklefs *et al.* 1998](#)) relative to body mass, and larger wing areas, compared with control fledglings. We have previously shown that *in ovo* exposure to corticosterone can have multiple downstream effects on size, growth and physiological pathways at fledging ([Love & Williams 2008a,b](#)). The underlying mechanisms by which embryonic exposure to maternal stress alters these pathways, as well as those of future muscle development and feather growth reported here, are not well investigated, and could include both direct and indirect developmental pathways. Direct mechanisms could include the known influence of glucocorticoids as transcription factors, given that many genes have glucocorticoid response elements, and thus embryonic exposure to elevated maternal stress hormones may have positive downstream effects on development (for review see [Byrne 2001](#)). Interestingly, however, these specific 'positive' effects of maternal glucocorticoids on muscle and feather development temporally overlap with the reported 'negative' effects of these hormones on size at hatching/birth and growth (see [Love *et al.* 2005](#); [Rubolini *et al.* 2005](#); [Saino *et al.* 2005](#); [Hayward *et al.* 2006](#); [Love & Williams 2008a](#)). However, these glucocorticoid-induced developmental processes are thought to occur through inhibition of cell proliferation and the growth of specific systems ([Orth *et al.* 1992](#)) via downregulation of growth hormone and insulin-like growth factor activity, as well as via a reduction in the ability to both self-regulate glucocorticoid receptors (see [Seckl 2004](#)) and interact with developmental thyroid hormones ([De Jesus *et al.* 1990](#); [Redding *et al.* 1991](#)). Indirect mechanisms of embryonic exposure to corticosterone could include changes in the allocation of resources within nestlings and changes in offspring behaviour brought about by the effects of embryonic exposure on the adrenocortical axis ([Love & Williams 2008b](#)), and even changes in the types of food brought to offspring by parents. Separating offspring from parents at hatch and raising nestlings under similar captive conditions will help us understand how embryonic developmental pathways are differentially responsive to the same degree of maternally derived stress, a fundamentally important step towards more fully appreciating how offspring interpret, and respond to, developmental stress.

Together with heavier and functionally more mature flight muscles, fledglings exposed to elevated corticosterone as embryos had correspondingly higher CS activity per gram of tissue (that is, functionally more mature tissue contained higher total enzymatic activity). Citrate synthase is a Krebs's cycle enzyme and its activity is used as both an index of mitochondrial density ([Guglielmo *et al.* 2002](#)) and oxidative capacity ([Hohtola & Visser 1998](#)). In muscle, CS activity has been correlated with measures of whole-body aerobic capacity (VO_{2max}) in a variety of taxa, including birds ([Hammond *et al.* 2000](#)), and CS activity is upregulated in adult birds in preparation for migration ([Lundgren & Kiessling 1985](#)). A more complete understanding of underlying mechanisms may be obtained by also considering anaerobic enzyme activity by examining CPK, since it catalyses the conversion of PCr to creatine and inorganic

phosphate (P_i) under anaerobic conditions. Since muscle PCr stores are rapidly depleted, hydrolysis of PCr can only be used to power very brief periods of high-intensity exercise and, as such, differences in CPK activity with treatment would probably be manifested in our measures of whole animal performance. Although marginally significant, CPK activity was higher in corticosterone-exposed fledglings than in controls, suggesting a biochemical basis for the performance differences we detected. The observation that enzyme activity differed with treatment when expressed per gram wet mass of tissue, but not when expressed per unit protein, is informative. It suggests that differences in activity with treatment were largely due to the functional maturity of the muscle (i.e. % water content). Thus nestlings did not adjust enzyme content independently of muscle functional maturity; corticosterone-exposed nestlings appeared simply to add more of the same basic components to build larger muscles.

(c) Positive effects of embryonic exposure to corticosterone

Many recent ecological studies examining the effects of maternal stress on offspring report negative developmental impacts on offspring phenotype ([Love *et al.* 2005](#); [Rubolini *et al.* 2005](#); [Saino *et al.* 2005](#); [Hayward *et al.* 2006](#); [Love & Williams 2008a](#)), although transient effects such as decreases in size and growth may have fitness benefits when viewed in the longer term ([Hayward *et al.* 2006](#); [Love & Williams 2008a](#)). Our results suggest that the negative impacts of maternal stress on offspring size, growth and immune function may be balanced by positive effects on offspring performance. Importantly, this work also emphasizes that embryonic exposure to the same elevated levels of yolk corticosterone can have differential effects on offspring phenotypic traits. Taken together, these results suggest that it is relevant to view effects of maternal stress on offspring in the longer term (from parental independence and beyond) to interpret how hormone-induced phenotypic flexibility might affect fitness. Moreover, while these current results provide tantalizing information indicating that maternally derived stress hormones may provide an adaptive signal to the developing offspring of the quality of its future environment (i.e. [Love *et al.* 2005](#); [Mathis *et al.* 2008](#)), it remains to be seen if embryonic exposure to glucocorticoids impacts offspring fitness ([Breuner 2008](#); [Love & Williams 2008a](#)).

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