

Determinants of within- and among-clutch variation in yolk corticosterone in the European starling

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Abstract

Maternal glucocorticoids are known to affect offspring phenotype in numerous vertebrate taxa. In birds, the maternal transfer of corticosterone to eggs was recently proposed as a hormonal mechanism by which offspring phenotype is matched to the relative quality of the maternal environment. However, current hypotheses lack supporting information on both intra- and inter-clutch variation in yolk corticosterone for wild birds. As such, we examined variation in yolk corticosterone levels in a wild population of European starlings (*Sturnus vulgaris*). Maternal condition, clutch size and nesting density were all negatively related to yolk corticosterone deposition; females with high condition indices, those laying larger clutches and those nesting in high-density associations deposited lower amounts of the hormone into eggs than those with low condition indices, laying small clutches and nesting in isolation. Alternatively, we found no effects of maternal age or human disturbance on yolk corticosterone deposition. Intra-clutch variation of yolk corticosterone was significant, with levels increasing across the laying sequence in all clutch sizes examined, with the difference between first and last-laid eggs being greater in large versus small clutches. Given the reported effects of yolk corticosterone on offspring size and growth, intra-clutch variation in yolk corticosterone has the potential to alter the competitive environment within a brood. Furthermore, our results indicate that variation in yolk corticosterone can originate from variation in both the mother's quality as well as the quality of her breeding environment. The presence of inter-female variation in particular is an important pre-requisite in testing whether the exposure of offspring to maternally-derived corticosterone is a mechanistic link between offspring phenotypic plasticity and maternal quality.

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Introduction

How and why maternal stress hormones affect offspring phenotype has become a subject of considerable interest in studies spanning numerous vertebrate taxa (e.g., Seckl, 2001; Cree et al., 2003; Love et al., 2005; Meylan and Clobert, 2005; Pike and Petrie, 2006; Uller and Olsson, 2006). In birds, corticosterone serves to mediate adaptive physiological and behavioral responses to 'stressful' events (Wingfield et al., 1998; Sapolsky et al., 2000; Wingfield, 2005) within the larger context of the hormone's role in maintaining daily homeostatic

energetic balance (Harvey et al., 1984; Dallman et al., 1993; Remege-Healey and Romero, 2001). In adult females, both short-term and life-history stage-related changes in corticosterone secretion may play adaptive roles (Wingfield et al., 1997; Moore and Jessop, 2003; Love et al., 2004) and baseline corticosterone can be influenced by social encounters (DeVries et al., 2003; Goymann and Wingfield, 2004). However, maternal glucocorticoids are also readily (apparently in direct relation to maternal plasma levels, see Love et al., 2005) transferred from mother to egg via the yolk during laying (McCormick, 1998; Hayward and Wingfield, 2004; Hayward et al., 2005; Love et al., 2005; Navara et al., 2006). Furthermore, embryos appear sensitive to maternal glucocorticoids during development and express many phenotypic changes in response to exposure in a wide range of vertebrate taxa: reduced body masses upon hatching or parturition, reduced postnatal growth rates and

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long-term ‘fetal programming’ effects on behavior and physiology (e.g., fish: McCormick, 1998, 1999; reptiles: Sinervo and DeNardo, 1996; Cree et al., 2003; Meylan and Clobert, 2005; birds: Hayward and Wingfield, 2004; Love et al., 2005; Rubolini et al., 2005; Saino et al., 2005; and mammals: review in Seckl, 2001). Given that mothers must balance multiple potential costs and benefits of corticosterone during reproduction (Love et al., 2004), it is important to understand why, in an evolutionary sense, mothers allow their offspring to be exposed to corticosterone via its transfer to the egg (Hayward and Wingfield, 2004; Love et al., 2005; Saino et al., 2005). To properly understand this, it is important to determine what causes variation in yolk corticosterone both across females and within clutches.

A decrease in the quality of many environmental variables is associated with elevated plasma levels of corticosterone in vertebrates: (1) low quality nutritional resources and declines in body condition (Holberton et al., 1996; Kitaysky et al., 1999a,b, 2001, 2006; Love et al., 2005), (2) poor quality habitats (Marra and Holberton, 1998; Suorsa et al., 2003; Kitaysky et al., 2006), (3) exposure to severe weather conditions (Wingfield et al., 1997; Romero et al., 2000; Breuner and Hahn, 2003), (4) increased predation risk (Boonstra et al., 1998; Cockrem and Silverin, 2002; Clinchy et al., 2004), (5) exposure to human disturbance (Fowler, 1999; Creel et al., 2002; Müllner et al., 2004; Walker et al., 2005; Lucas et al., 2006; Pereira et al., 2006) and (6) exposure to socially dominant conspecifics (Creel et al., 1996; DeVries et al., 2003; Goymann and Wingfield, 2004). With this information as a basis, it has been suggested that the phenotypic effects of maternal glucocorticoids on offspring may be a mechanistic link between maternal and offspring quality (de Fraipont et al., 2000; Seckl, 2001; Love et al., 2005; Meylan and Clobert, 2005). For example, using an experimental field study in laying wild European starlings (*Sturnus vulgaris*), Love et al. (2005) found that the transfer of maternal corticosterone to yolks resulted in female-biased sex ratios and lower male quality at hatching and during development. The authors suggested that yolk corticosterone may represent a mechanistic link between maternal quality and sex-biased maternal investment in offspring, a potentially ubiquitous sex allocation mechanism across avian species (Pike and Petrie, 2003, 2005a, 2005b). Alternatively, the maternal transfer of corticosterone to offspring may instead represent a developmental cost to the offspring that mothers cannot avoid (Rubolini et al., 2005; Saino et al., 2005; Janczak et al., 2006) due to hormonal constraints (see Ketterson and Nolan, 1999). This could arise from both the potential pleiotropic effects of hormones (Ketterson and Nolan, 1999; Williams, 2005) and that mothers may not be able to buffer eggs from high maternal plasma levels of corticosterone (Saino et al., 2005; although see Cree et al., 2003; Love et al., 2005).

In this study, we measured intra- and inter-clutch variation in yolk corticosterone levels in eggs of wild European starlings, a facultatively polygynous cavity-nesting bird (Cabe, 1993). We predicted significant inter-female differences in yolk corticosterone (representing inherent variation in both the internal and external maternal environment). More specifically, since yolk

corticosterone can be negatively related to maternal condition in starlings through a direct relationship with maternal plasma corticosterone (Love et al., 2005), we predicted that traits related to maternal quality (i.e., maternal condition, clutch size, age) and the quality of the breeding environment (degree of human disturbance, nesting density) would be correlated with yolk corticosterone levels (e.g., positive for nesting density and human disturbance and negative for age, clutch size and maternal condition). Furthermore, based on the hypothesized cumulative energetic costs for the formation and maintenance of the reproductive machinery and the developing follicles in egg-producing female passerines (e.g., Nilsson and Råberg, 2001; Vézina and Williams, 2002; Vézina et al., 2003), we also predicted an increase in yolk corticosterone levels across the laying sequence due to potential increases in maternal plasma corticosterone across the laying sequence.

Methods

Study species, site and egg collection

Research was carried out at the Davistead Dairy Farm in Langley, British Columbia, Canada (49°10' N, 122°50' W), a site consisting of 225 nest boxes used yearly by breeding starlings. All work was conducted between April and the beginning of June (the breeding period within which pairs successfully fledge at least one nestling) of 2004 under a Simon Fraser University Animal Care permit (657B-96), following guidelines of the Canadian Council on Animal Care. Starlings at this site lay 5.9 ± 0.2 (mean \pm SEM) eggs per clutch within the synchronous peak of laying (covers 7–8 days; Love et al., 2005; Smith, 2004), incubate for 10.3 ± 0.1 days and fledge nestlings 21 ± 0.6 days following hatching (Love et al., 2005). Nest boxes were checked daily to determine clutch initiation and clutch completion dates as well as the laying sequence of eggs. To examine both intra- and inter-individual variation in yolk corticosterone levels, freshly laid eggs for a given clutch were collected daily (within 2–5 h of being laid) when females were foraging away from their nests (in order to eliminate possible disturbance effects on yolk corticosterone levels in subsequent eggs) and replaced with dummy eggs for 30 individual female starlings. Fresh eggs were marked and measured and immediately stored whole at -20 °C until further analysis, as suggested in Eising et al. (2001). These starlings ($n=29$; one could not be caught) were captured 2–3 days after completing their clutch of eggs while roosting in their nest box at night (usually between 2000 and 2400 h) when baseline corticosterone levels in starlings are at their daily mean with respect to daily variation (Romero and Remage-Healey, 2000). Since corticosterone increases rapidly in birds following capture (Romero and Romero, 2002), we blood sampled all birds from the wing vein within 2 min of capture to insure we measured baseline levels. We detected no effect of time after capture (within 0–2 min interval) on baseline corticosterone levels in initial blood samples ($R^2=0.05$, $P=0.86$) and therefore samples were considered to reflect baseline corticosterone levels. Birds were subsequently weighed, measured (exposed culmen, flattened wing cord and tarsus) and metal-banded (permit #10646) for individual identification. A throat feather was also removed to age females (either hatch year or after-hatch year) based on the length of the iridescent portion of each feather following Pyle (1997).

Plasma and yolk corticosterone determination

The concentration of total corticosterone (see Breuner and Orchinik, 2002; Love et al., 2004, 2005) in both plasma and yolks was determined using a corticosterone EIA (0.31% cross-reactivity with testosterone; Assay Designs Inc., MI, USA) with a 4-parameter logistic fit. Assay sensitivity was 32 through 20,000 pg/well and all determinations fell within this range. Yolks were bisected, making sure to include all yolk layers, and were then extracted prior to assay by the methods outlined in Love et al. (2005). Briefly, following equilibration with distilled/deionized (dd) water, dilution with distilled-in-glass

Table 1
Statistical relationship between residual yolk corticosterone levels and various traits measured in European starlings

Trait	df	F	P	B	SE (B)
Laying order	5,54	34.3	<0.0001	2.46	0.29
Clutch size (4)	2,11	7.5	<0.01	1.68*	0.46
Clutch size (5)				1.97*	0.37
Clutch size (6)				0*	0
Maternal condition	1,11	9.36	<0.01	-3.56	1.23
Nesting density	5,6	4.6	<0.05	-1.16	0.49

*Slope when compared to a clutch size of six eggs.

(DIG) methanol and centrifugation at 1500×g for 7 min under refrigeration (2±1.5 °C), supernatant was extracted on C18 columns (IS2200050C Isolute® SPE columns, Chromatographic Specialties, Inc., Brockville, ON) under vacuum filtration. Columns were primed with DIG methanol, followed by dd water, followed by the entire 10 ml sample volume, and then washed with dd water. Corticosterone was eluted with 5 ml of 90% methanol into 7 ml borosilicate vials (03-337-26, Fisher). Each sample was evaporated to dryness under a stream of air and reconstituted in 1.2 ml of assay buffer (5% ethanol) prior to being quantified in triplicate on a single plate of the corticosterone EIA. As internal controls, seven additional yolks were used to quantify extraction efficiency. The seven yolks were mixed by hand using a mortar and pestil on dry ice and 12 fractions were weighed from the combined yolk and thereafter treated as independent yolk samples. After dilution into a total volume of 2 ml, six of the 12 samples were spiked with a 5 ng/ml bolus of corticosterone in a 200 µl volume drawn from commercial standards from a Corticosterone I¹²⁵ RIA kit (ICN, Orangeburg, NY) and the remaining six samples were spiked with 200 µl of assay diluent from the same kit. The resulting samples settled overnight and were handled exactly the same way as the unknown yolk determinations so that the controls contained raw yolk, or raw yolk plus 5 ng of corticosterone. Each assay contained three replicates of both the un-spiked control and spiked yolks for the correct calculation of both intra- and inter-assay variation, respectively. Corticosterone concentration in the raw yolk was 15.25±0.64 ng/g (mean±SEM), with an average intra-assay variation of 7.6% and an average inter-assay variation of 8.9%. Average recovery of the 5 ng spike in the six replicates was 94.6±2.9%. Values are reported as corrected for the recovery efficiency.

Statistical analyses

We used a GLMM (General Linear Mixed Model) to analyze variation in yolk corticosterone by including maternal age, clutch size and human disturbance as fixed factors and laying order, maternal body condition index and nesting density index as covariates; maternal identity was included as a random factor. Given the previously reported relationship between maternal plasma and yolk corticosterone in this species and the hypothesis that maternal levels are thought to directly influence yolk levels (Love et al., 2005), we thought it prudent to correct yolk corticosterone values for the influence of maternal levels (residual yolk corticosterone) rather than merely include maternal plasma corticosterone as another independent variable in the analysis. However, we present results for both corrected and absolute yolk corticosterone levels in the results section. Furthermore, we carried out these analyses for both (1) the concentration of corticosterone per gram of yolk and (2) total yolk corticosterone per egg to account for potential differences between absolute concentrations and hormone levels that particular nestlings may be exposed to, respectively. We focused on yolk rather than egg mass since yolk mass is highly correlated with egg mass in our population (linear regression analysis: $R^2=0.96$, $n=196$ eggs from 48 clutches) and is the focus target of hormone deposition in this study. We used the REstricted Maximum Likelihood (REML) method in JMP 6.0 (SAS Inc., 2006) and all possible interactions (three- and two-way) between factors and covariates were initially included in the model. For comparison of the percent difference in yolk corticosterone between first- and last-laid eggs, we first transformed the proportion data using an arcsine-root transformation (since the proportion data were not normally distributed) and then used a one-way ANOVA to analyze for differences between clutch sizes.

Maternal body condition index (body mass corrected for body size) was calculated by taking the residuals of a linear regression between body mass against the first principal component score (PC1) calculated from a principal-component analysis (PCA) for body size based on exposed culmen, tarsus and flattened wing cord as described in Love et al. (2005). Nesting density index was calculated as the total number of active nests (nest with a concurrently laying female present within a radius of 10 m from the focal nest box) given that nesting density has been reported to influence yolk androgen deposition through potential inter-female interactions (Groothuis and Schwabl, 2002; Pilz and Smith, 2004). Nest boxes were categorized as being located in one of two locations on the farm receiving different degrees of daily human disturbance (i.e., either within the core area of the working farm and at a dairy barn visited daily by farm workers or in field locations away from any human contact). Although the *a priori* expectation was that nest boxes used in the human-associated farm areas would experience more human activity than boxes mounted in field locations, we used behavioral observations (as part of ongoing provisioning studies) during the 2002, 2003 and 2004 field seasons to confirm this prediction. Observations were made over three consecutive days when boxes in these areas contained nestlings aged 6–10 days (see Chin et al., 2005; Love et al., 2005), although daily human activity patterns at the farm do not change during the period when birds are laying to when nestlings are fledging.

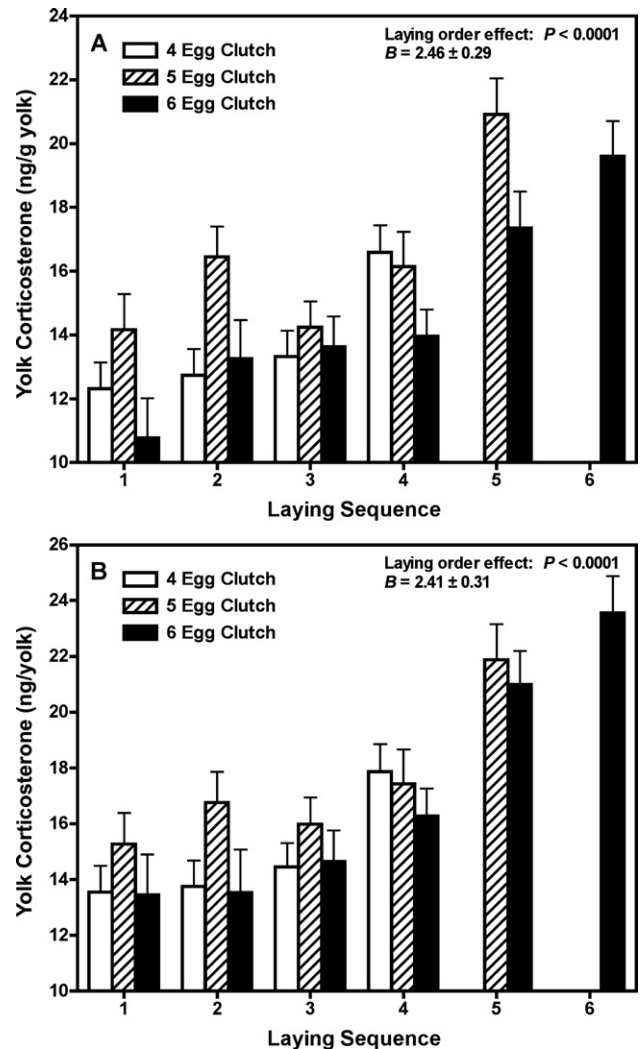


Fig. 1. Laying sequence-specific patterns of yolk corticosterone levels (ng/g of yolk) (A) and total yolk corticosterone (ng per yolk) (B) in European starling eggs in relation to clutch size (mean±SEM are shown); laying order effects for both dependent variables $P < 0.0001$, B is the slope of the laying order effect across the laying sequence±SEM.

Observations from boxes located in human-associated areas ($n=143$) and in field locations ($n=86$) confirmed that the former experienced significantly more hours of daily human disturbance (ANCOVA: $F_{1,221}=5.65$, $P=0.018$, controlling for laying date). As such, we felt confident in identifying these two areas as distinct categories of human disturbance levels. All reported means are given as the mean \pm SEM.

Results

Yolk corticosterone concentrations (ng/g of yolk) showed a normal distribution within the clutches examined (Shapiro–Wilk W test, controlling for nest origin; $w=0.74$, $P=0.62$; $n=148$ eggs from 30 nests) with the mean level of yolk corticosterone across females being 15.34 ± 0.66 ng/g of yolk. Average yolk corticosterone in eggs was significantly negatively related to maternal plasma corticosterone at clutch completion ($F_{1,25}=4.53$, $P<0.05$, $b=-0.53$). Although results are similar whether absolute or plasma-corrected yolk corticosterone was included as the dependent variable, we report results for both to be conservative; figures for corrected yolk corticosterone are reported because this dependent variable produced the strongest model (see below). We found no effects of laying order on yolk mass ($F_{5,52}=1.02$, $P=0.42$), as such it is not surprising that similar significant overall models were produced regardless of whether the dependent variable was the concentration of corticosterone per gram of yolk or total yolk corticosterone per egg; we therefore report results for the former here as this makes comparisons across studies possible. This revealed a significant model (adjusted $R^2=0.77$) with 24.1% of the variance in residual yolk corticosterone contributed by individual female component and 75.9% by the independent variables component in the model; for absolute yolk corticosterone levels, the best model produced an adjusted $R^2=0.67$, with 27.2% of the variance in yolk corticosterone contributed by individual female component and 72.8% by the independent variables. We found a strong effect of laying order ($F_{5,54}=34.3$, $P<0.0001$; $b=2.46$, Table 1, Fig. 1), clutch size ($F_{2,11}=7.5$, $P<0.01$; $b=1.68$ for 4 eggs, $b=1.97$ for 5 eggs and $b=0$ for 6 eggs, Table 1, Fig. 2), maternal condition index ($F_{1,11}=9.36$,

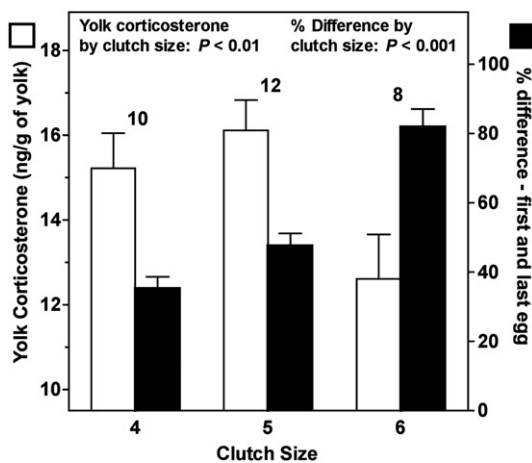


Fig. 2. Mean yolk corticosterone levels (ng/g yolk) and percent difference in yolk corticosterone between first- and last-laid eggs of European starling eggs in the three clutch sizes examined in this study (numbers over bars represent number of clutches used, $n=30$ clutches in total and mean \pm SEM are shown).

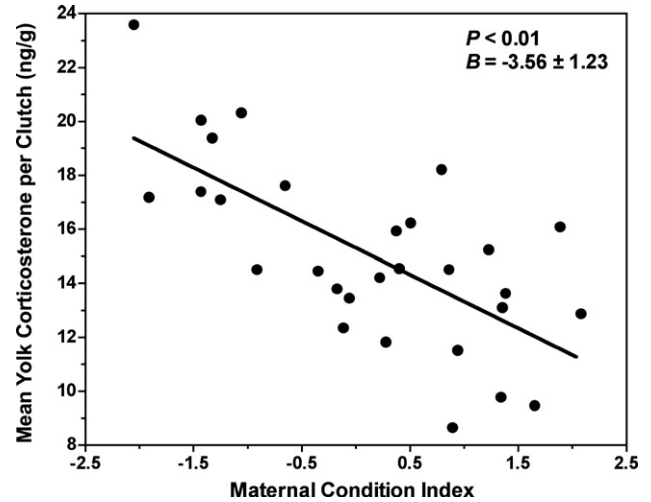


Fig. 3. Relationship between maternal condition and mean yolk corticosterone levels (ng/g of yolk) per clutch for individual female European starlings ($n=29$ clutches; $P<0.01$, B is the slope of the maternal condition relationship \pm SEM).

$P=0.01$; $b=-3.56$, Table 1, Fig. 3) and nesting density ($F_{5,6}=4.6$, $P<0.05$; $b=-1.16$, Table 1, Fig. 4) on residual yolk corticosterone levels. Results were very similar for absolute yolk corticosterone, with a strong effect of laying order ($F_{5,54}=23.4$, $P<0.0001$; $b=2.41$), clutch size ($F_{2,11}=7.1$, $P=0.01$; $b=1.61$ for 4 eggs, $b=1.81$ for 5 eggs and $b=0$ for 6 eggs), maternal condition index ($F_{1,11}=7.62$, $P=0.02$; $b=-2.14$) and nesting density ($F_{5,6}=4.4$, $P=0.05$; $b=-1.48$). Neither maternal age nor human disturbance index showed any relationship to yolk corticosterone levels (residual yolk corticosterone: both $P>0.70$; absolute yolk corticosterone: both $P>0.81$) and we found no significant interactions of any kind between factors or covariates for either dependent variable. Finally, we found that the difference in both residual and absolute yolk corticosterone between the first- and

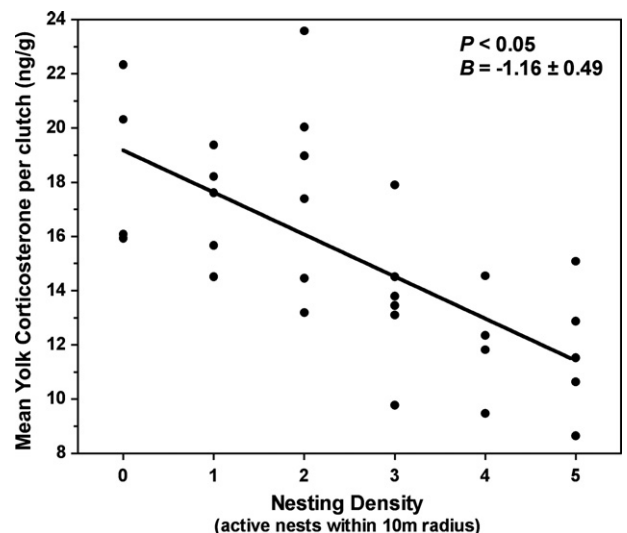


Fig. 4. Relationship between nesting density and mean yolk corticosterone levels (ng/g of yolk) per clutch for individual female European starlings ($n=30$ clutches; $P<0.05$, B is the slope of the nesting density relationship \pm SEM).

Table 2
Corticosterone levels (from yolk and albumin) in the eggs of avian species

Species	Population	Source	Levels (mean±SEM)	Reference
Chicken (<i>Gallus domesticus</i>)	Domestic	Albumin	1.2±NA ng/ml	1
Chicken (<i>Gallus domesticus</i>)	Domestic	Yolk	1.12±0.15 ng/ml	2
Japanese quail (<i>Coturnix c. japonica</i>)	Domestic	Yolk	1.11±0.30 ng/g	3
Canary (<i>Serinus canaria</i>)	Domestic	Yolk	<0.5 ng/yolk	4
Zebra finch (<i>Taeniopygia guttata</i>)	Domestic	Yolk	<0.1 ng/yolk	4
Peafowl (<i>Pavo cristatus</i>)	Captive	Yolk	32.2±2.3 ng/g	5
Eastern bluebird (<i>Sialia sialis</i>)	Wild	Yolk	2.5±0.75 ng/g	6
European starling (<i>Sturnus vulgaris</i>)	Wild	Yolk	14.13±2.31 ng/g	7
European starling (<i>Sturnus vulgaris</i>)	Wild	Yolk	15.34±0.66 ng/g	8
Barn swallow (<i>Hirundo rustica</i>)	Wild	Albumin	1.1±0.8 ng/ml	9
Yellow-legged gull (<i>Larus michahellis</i>)	Wild	Albumin	1.86±0.13 ng/g	10

References: (1) Eriksen et al., 2003, (2) Janczak et al., 2006, (3) Hayward et al., 2006, (4) Schwabl, 1993, (5) Pike and Petrie, 2005b, (6) Navara et al., 2006, (7) Love et al., 2005, (8) Present study, (9) Saino et al., 2005, (10) Rubolini et al., 2005.

last-laid egg in a clutch was greater in larger versus smaller clutches (residual: $F_{2,15}=11.7$, $P<0.001$, Fig. 2; absolute: $F_{2,15}=9.5$, $P=0.002$).

Discussion

Data on yolk corticosterone have been published for seven avian species to date, four domestic, one captive and only two wild; data on albumin corticosterone have been reported for three species, one domestic and two wild (Table 2). Yolk corticosterone levels in the domestic species are relatively low compared to the reported levels for captive and wild species. Unfortunately, this species sample is biased toward the *Galliformes* and especially toward human-exposed animals that generally show lower levels of plasma corticosterone due to potential habituation effects on the stress axis (see Romero and Wikelski, 2002; Love et al., 2003; Walker et al., 2006).

We found a negative relationship between plasma and yolk corticosterone, with mothers that had deposited the highest levels into eggs having the lowest plasma levels by clutch completion. Although we have previously reported a positive relationship between these two traits in this species when comparing maternal plasma corticosterone collected at the beginning of laying to the yolk corticosterone deposited in the first-laid egg (Love et al., 2005), the plasma samples in the present study were collected when the clutch had been completed. In support of the present findings, the same negative relationship between yolk testosterone and maternal plasma testosterone sampled at clutch completion was reported recently in house sparrows (*Passer domesticus*) by Mazuc et al. (2003). Recently, Navara and colleagues (2006) proposed a mechanism for this relationship in that the yolk may act as a reservoir for the deposition of maternal-derived steroid hormones. If this is possible, it suggests that mothers depositing high levels into yolks may be left with a temporary deficit of the hormone in the plasma immediately following laying. Alternatively, since elevated plasma corticosterone is controlled through a negative feedback pathway (Romero, 2004), elevated plasma levels during laying may be decreased through this feedback by clutch completion. Although more work will be needed to examine this phenomenon more carefully, our results combined with

those of other wild species indicate that significant amounts of corticosterone can be transferred from mother to offspring via the yolk. However, a more varied examination of inter-specific variation in yolk corticosterone levels will be necessary to separate phylogenetic versus methodological differences between studies.

Inter-female variation in yolk corticosterone: condition and social influences

Maternally derived yolk corticosterone has been hypothesized to provide a hormonal mechanism by which mothers can adjust investment in offspring to match maternal and/or environmental quality (Love et al., 2005). For this to be the case, the quality of the mother and the environment she reproduces in should correlate with corticosterone levels deposited in the egg, of which there is already some evidence (Love et al., 2005; Saino et al., 2005). We predicted that traits predicted to correlate with maternal quality would be negatively related to yolk corticosterone deposition. Indeed, mothers with low condition indices laid eggs with high levels of yolk corticosterone. These results support those of Love et al. (2005) who found that low maternal condition was correlated with high maternal plasma corticosterone measured at the first-egg stage and that females with experimentally elevated plasma corticosterone transferred elevated corticosterone levels to yolks. We also found that yolks contained lower levels of corticosterone in large versus smaller clutches and this is in concordance with the finding that in starlings high quality mothers produce the largest clutches (Christians et al., 2001). However, we did not find a relationship between maternal age and yolk corticosterone as previously reported for yolk androgens in this species (Pilz et al., 2003), nor did we find an effect of human disturbance on yolk corticosterone as recently reported by Saino et al. (2005) in barn swallows (*Hirundo rustica*). These results may be due to the combination of the overriding influences of maternal condition and clutch size rather than age *per se* on yolk corticosterone deposition, as well as the potential predictability of the human disturbance by birds nesting in these locations. Indeed, whereas Saino and colleagues examined relatively undisturbed birds following an acute experimental disturbance,

the starlings in the high-human disturbance areas had already chosen to nest in these locations. In other words, we worked with individuals of a species known to be well adapted to chronic and predictable disturbance and therefore they may show little or no effects of the chronic disturbance compared with the acute effects on the stress axis reported in other species (see Introduction).

We initially predicted that the type of social environment a mother reproduces in would be positively related to yolk corticosterone deposition, with higher nesting density resulting in more aggressive interactions and therefore higher yolk corticosterone levels. Indeed, females nesting in higher densities or those experiencing high levels of inter-female aggressive interactions lay eggs with elevated yolk androgens in a number of avian species (Schwabl, 1997; Groothuis and Schwabl, 2002; Wittingham and Schwabl, 2002; Pilz and Smith, 2004; Navara et al., 2006). However, female starlings nesting in high density associations deposited less corticosterone into eggs than females nesting in isolation, independent of clutch size and maternal condition (two measures of quality). Although it is well known that dominance hierarchies in social species can result in higher levels of glucocorticoids in individuals (Creel et al., 1996; DeVries et al., 2003; Goymann and Wingfield, 2004), European starlings are a semi-colonial-nesting species (Cabe, 1993). As such, higher nest-site densities may in fact be preferable to starlings by potentially decreasing adult predation risk through increased group vigilance (see Krebs and Davies, 1987). In captive starlings, exposure of a resident bird to a conspecific intruder does not result in an increase in plasma corticosterone in the resident (Nephew and Romero, 2003). Furthermore, an experimental nest intrusion experiment in wild eastern bluebirds did not result in higher yolk corticosterone levels (Navara et al., 2006). If higher nesting density results in lower adult predation risk, adult laying females may be less physiologically 'stressed' given that interaction with predators can increase both plasma corticosterone in adults (Cockrem and Silverin, 2002; Clinchy et al., 2004) and albumin corticosterone in eggs (Saino et al., 2005). Indeed, there is some evidence that in colonial and communally nesting species, mothers can change egg quality depending on their perception of the quality of the social environment (Macedo et al., 2004; Verboven et al., 2005). Our results on inter-individual differences in yolk corticosterone deposition suggest that there can be both a significant maternal quality and surrounding maternal environmental quality component contributing to variation in the yolk corticosterone.

Intra-clutch variation and implications for offspring

Laying sequence data in starlings suggest that yolk corticosterone varies significantly across eggs within a clutch, a trait that has received significant attention in research on variation in yolk androgens (review in Groothuis et al., 2005; Badyaev et al., 2006). Our results indicate that the difference in yolk corticosterone between first- and last-laid eggs was as little as 35% in four-egg clutches and as much as 82% in six-egg clutches. The result would be a greater disparity in yolk corticosterone between first- and last-laid eggs in large compared with small clutches. Embryos

developing from later-laid eggs are therefore exposed to much higher yolk corticosterone levels than their earlier-laid siblings. Given the well documented effects of maternally transferred egg corticosterone on offspring phenotype in birds (Eriksen et al., 2003; Hayward and Wingfield, 2004; Love et al., 2005; Rubolini et al., 2005; Saino et al., 2005; Janczak et al., 2006; Hayward et al., 2006), it is plausible to predict significant hormone-mediated disparity in the quality and phenotype of siblings within a brood (Love et al., 2005; Saino et al., 2005). For example, nestlings from later-laid eggs exposed to high levels of the hormone may be smaller at hatching and potentially more likely to be out-competed by larger offspring hatched from earlier-laid eggs (see Love et al., 2005). The result would be a hormonal mechanism mediating brood reduction when environmental conditions during chick-rearing are poor or in habitats where the correlation between environmental quality at laying and conditions during chick-rearing might be low and unpredictable (Nager et al., 2000). Those females with large brood sizes would especially benefit from a rapid mechanism of brood reduction (i.e., Lack, 1947, 1954) if environmental quality decreased from laying to hatching, allowing a mother to maximize the quality of the young she does fledge. Practically speaking, it is difficult at this point to determine the source of such intra-clutch variation in yolk corticosterone. One possible 'passive' source is the potential cumulative stress of egg-laying through either the direct energetic costs of forming and maintaining the reproductive machinery, developing follicles and eggs (Nilsson and Råberg, 2001; Vézina and Williams, 2002; Vézina et al., 2003), or the indirect costs associated with increased predation risk from elevated body weight due to the added mass of the reproductive machinery during the laying period (Lee et al., 1996; Kullberg et al., 2002, 2005). Since plasma glucocorticoids are tightly tied to homeostatic energy balance (Harvey et al., 1984; Dallman et al., 1993; Remage-Healey and Romero, 2001), an increase in maternal energetic deficits across laying may hormonally manifest itself in both increasing maternal plasma and yolk corticosterone levels across the laying sequence. Maternal 'control' over these intra-clutch patterns (see Groothuis et al., 2005 for a review of the debate on maternal control) is therefore not a prerequisite for intra-clutch variation in yolk corticosterone to be adaptive from the mother's standpoint. Mothers laying larger clutches deposit more hormone into later-laid eggs (as shown in the present study) and the difference between first- and last-laid eggs would hypothetically benefit these mothers during periods when offspring competition is intense through rapid, yolk corticosterone-mediated brood reduction. Although this and many other questions remain regarding the evolutionary role of maternally derived yolk glucocorticoids, their transfer to eggs provides an exciting system in which to examine the relationship between maternal quality and phenotypic plasticity of offspring.

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References

- Badyaev, A.V., Acevedo-Seaman, D., Navara, K.J., Hill, G.E., Mendonça, M.T., 2006. Evolution of sex-biased maternal effects in birds: III. Adjustment of ovulation order can enable sex-specific allocation of hormones, carotenoids, and vitamins. *J. Evol. Biol.* 19, 1044–1057.
- Boonstra, R., Hik, D., Singleton, G.R., Tinnikov, A., 1998. The impact of predator-induced stress on the snowshoe hare cycle. *Ecol. Monogr.* 79, 371–394.
- Breuner, C.W., Orchinik, M., 2002. Beyond carrier proteins: plasma binding proteins as mediators of corticosteroid action in vertebrates. *J. Endocrinol.* 175, 99–112.
- Breuner, C.W., Hahn, T.P., 2003. Integrating stress physiology, environmental change, and behavior in free-living sparrows. *Horm. Behav.* 43, 115–123.
- Cabe, P.R., 1993. European starling (*Sturnus vulgaris*). *The Birds of North America*. No 48 (eds. A. Poole and F. Gill), Philadelphia: The Academy of Natural Sciences; Washington, D.C.: The American Ornithologists' Union.
- Chin, E.H., Love, O.P., Clark, A.M., Williams, T.D., 2005. Brood size and environmental conditions sex-specifically affect nestling immune response in the European starling *Sturnus vulgaris*. *J. Avian Biol.* 36, 549–554.
- Christians, J.K., Evanson, M., Aiken, J.J., 2001. Seasonal decline in clutch size in European starlings: a novel randomization test to distinguish between the timing and quality hypotheses. *J. Anim. Ecol.* 70, 1080–1087.
- Clinchy, M., Zanette, L., Boonstra, R., Wingfield, J.C., Smith, J.N.M., 2004. Balancing food and predator pressure induces chronic stress in songbirds. *Proc. R. Soc. Lond., B* 271, 2473–2479.
- Cockrem, J.F., Silverin, B., 2002. Sight of a predator can stimulate a corticosterone response in the great tit (*Parus major*). *Gen. Comp. Endocrinol.* 125, 248–255.
- Creel, S., Creel, N.M., Monfort, S.L., 1996. Social stress and dominance. *Nature* 379, 212.
- Creel, S., Fox, J.E., Hardy, A., Sands, J., Garrott, R., Peterson, R., 2002. Snowmobile activity and glucocorticoid stress responses in wild wolves and elk. *Conserv. Biol.* 16, 809–814.
- Cree, A., Tyrrell, C.L., Preest, M.R., Thorburn, D., Guillelte Jr., L.J., 2003. Protecting embryos from stress: corticosterone effects and the corticosterone response to capture and confinement during pregnancy in a live-bearing lizard (*Hoplodactylus maculatus*). *Gen. Comp. Endocrinol.* 134, 316–329.
- Dallman, M.F., Strack, A.M., Akana, S.F., Bradbury, M.J., Hanson, E.S., Scribner, K.A., Smith, M., 1993. Feast and famine: critical role of glucocorticoids with insulin in daily energy flow. *Front. Neuroendocrinol.* 14, 303–347.
- de Fraipont, M., Clobert, J., John-Alder, H., Meylan, S., 2000. Increased prenatal maternal corticosterone promotes philopatry of offspring in common lizards *Lacerta vivipara*. *J. Anim. Ecol.* 69, 404–413.
- DeVries, A.C., Glasper, E.R., Detillion, C.E., 2003. Social modulation of stress responses. *Physiol. Behav.* 79, 399–407.
- Eising, C.M., Eikenaar, C., Schwabl, H., Groothuis, T.G.G., 2001. Maternal androgens in Black-headed gulls (*Larus ridibundus*) eggs: consequences for chick development. *Proc. R. Soc. Lond., B* 268, 839–846.
- Eriksen, M.S., Haug, A., Torjesen, P.A., Bakken, M., 2003. Prenatal exposure to corticosterone impairs embryonic development and increases fluctuating asymmetry in chickens (*Gallus gallus domesticus*). *Br. Poult. Sci.* 44, 690–697.
- Fowler, G.S., 1999. Behavioral and hormonal responses of Magellanic penguins (*Spheniscus magellanicus*) to tourism and nest site visitation. *Biol. Conserv.* 90, 143–149.
- Goymann, W., Wingfield, J.C., 2004. Allostatic load, social status and stress hormones: the costs of social status matter. *Anim. Behav.* 67, 591–602.
- Groothuis, T.G.G., Schwabl, H., 2002. Determinants of within- and among-clutch variation in levels of maternal hormones in Black-Headed Gull eggs. *Funct. Ecol.* 16, 281–289.
- Groothuis, T.G.G., Müller, W., Engelhardt von, N., Carere, C., Eising, C.M., 2005. Maternal hormones as a tool to adjust offspring phenotype in avian species. *Neurosci. Biobehav. Rev.* 29, 329–352.
- Harvey, S., Phillips, J.G., Rees, A., Hall, T.R., 1984. Stress and adrenal function. *J. Exp. Zool.* 232, 633–645.
- Hayward, L.S., Wingfield, J.C., 2004. Maternal corticosterone is transferred to avian yolk and may alter offspring growth and adult phenotype. *Gen. Comp. Endocrinol.* 135, 365–371.
- Hayward, L.S., Satterlee, D., Wingfield, J.C., 2005. Japanese quail selected for high plasma corticosterone response deposit high levels of corticosterone in their eggs. *Physiol. Biochem. Zool.* 78, 1026–1031.
- Hayward, L.S., Richardson, J.B., Grogan, M.N., Wingfield, J.C., 2006. Sex differences in the organizational effects of corticosterone in the egg yolk of quail. *Gen. Comp. Endocrinol.* 146, 144–148.
- Holberton, R.L., Parrish, J.D., Wingfield, J.C., 1996. Modulation of the adrenocortical stress response in neotropical migrants during autumn migration. *Auk* 113, 558–564.
- Janczak, A.M., Braastad, B.O., Bakken, M., 2006. Behavioural effects of embryonic exposure to corticosterone in chickens. *Appl. Anim. Behav. Sci.* 96, 69–82.
- Ketterson, E.D., Nolan, V., 1999. Adaptation, exaptation, and constraint: a hormonal perspective. *Am. Nat.* 154, S4–S25.
- Kitaysky, A.S., Wingfield, J.C., Piatt, J.F., 1999a. Dynamics of food availability, body condition and physiological stress response in breeding black-legged kittiwakes. *Funct. Ecol.* 13, 577–584.
- Kitaysky, A.S., Piatt, J.F., Wingfield, J.C., Romano, M., 1999b. The adrenocortical stress-response of black-legged kittiwake chicks in relation to dietary restrictions. *J. Comp. Physiol.* 169, 303–310.
- Kitaysky, A.S., Kitaiskaia, E.V., Wingfield, J.C., Piatt, J.F., 2001. Dietary restriction causes chronic elevation of corticosterone and enhances stress response in red-legged kittiwake chicks. *J. Comp. Physiol., B* 171, 701–709.
- Kitaysky, A.S., Kitaiskaia, E.V., Wingfield, J.C., Piatt, J.F., 2006. A mechanistic link between chick diet and decline in seabirds? *Proc. R. Soc. Lond., B* 273, 445–450.
- Krebs, J.R., Davies, N.B., 1987. *An Introduction to Behavioural Ecology*. Sinauer Associates, Maryland.
- Kullberg, C., Houston, D.C., Metcalfe, N.B., 2002. Impaired flight ability—a cost of reproduction in female blue tits. *Behav. Ecol.* 13, 575–579.
- Kullberg, C., Jakobsson, S., Kaby, U., Lind, J., 2005. Impaired flight ability prior to egg-laying: a cost of being a capital breeder. *Funct. Ecol.* 19, 98–101.
- Lack, D., 1947. The significance of clutch size. *Ibis* 89, 302–352.
- Lack, D., 1954. *The Natural Regulation of Animal Numbers*. Clarendon Press, Oxford.
- Lee, S.J., Witter, M.S., Cuthill, I.C., Goldsmith, A.R., 1996. Reduction in escape performance as a cost of reproduction in gravid starlings, *Sturnus vulgaris*. *Proc. R. Soc. Lond., B* 263, 619–624.
- Love, O.P., Shutt, L.J., Silfies, J.S., Bird, D.M., 2003. Repeated restraint and sampling results in reduced corticosterone levels in developing and adult captive American kestrels (*Falco sparverius*). *Physiol. Biochem. Zool.* 76, 753–761.
- Love, O.P., Breuner, C.W., Vézina, F., Williams, T.D., 2004. Mediation of a corticosterone-induced reproductive conflict. *Horm. Behav.* 46, 59–65.
- Love, O.P., Chin, E.H., Wynne-Edwards, K.E., Williams, T.D., 2005. Stress hormones: a link between maternal condition and sex-biased reproductive investment. *Am. Nat.* 166, 751–766.
- Lucas, J.R., Freeberg, T.M., Egbert, J., Schwabl, H., 2006. Fecal corticosterone, body mass, and caching rates of Carolina chickadees (*Poecile carolinensis*) from disturbed and undisturbed sites. *Horm. Behav.* 49, 634–643.
- Macedo, R.H.F., Cariello, M.O., Pacheco, A.M., Schwabl, H.G., 2004. Significance of social parameters on differential nutrient investment in guira cuckoo, *Guira guira*, eggs. *Anim. Behav.* 68, 685–694.

- Marra, P.P., Holberton, R.L., 1998. Corticosterone levels as indicators of habitat quality: effects of habitat segregation in a migratory bird during the non-breeding season. *Oecologia* 116, 284–292.
- Mazuc, J., Bonneaud, C., Chastel, O., Sorci, G., 2003. Social environment affects female and egg testosterone levels in the house sparrow (*Passer domesticus*). *Ecol. Lett.* 6, 1084–1090.
- McCormick, M.I., 1998. Behaviorally induced maternal stress in a fish influences progeny quality by a hormonal mechanism. *Ecology* 79, 1873–1883.
- McCormick, M.I., 1999. Experimental test of the effect of maternal hormones on larval quality of a coral reef fish. *Oecologia* 118, 412–422.
- Meylan, S., Clobert, J., 2005. Is corticosterone-mediated phenotype development adaptive? Maternal corticosterone treatment enhances survival in male lizards. *Horm. Behav.* 48, 44–52.
- Moore, I.T., Jessop, T.S., 2003. Stress, reproduction, and adrenocortical modulation in amphibians and reptiles. *Horm. Behav.* 43, 39–47.
- Müllner, A., Linsenmair, K.E., Wikelski, M., 2004. Exposure to ecotourism reduces survival and affects stress response in Hoatzin chicks (*Opisthocomus hoazin*). *Biol. Conserv.* 118, 549–558.
- Nager, R.G., Monaghan, P., Houston, D.C., Genovart, M., 2000. Parental condition, brood sex ratio and differential young survival: an experimental study in gulls (*Larus fuscus*). *Behav. Ecol. Sociobiol.* 48, 452–457.
- Navara, K.J., Siefferman, L.M., Hill, G.E., Mendonça, M.T., 2006. Yolk androgens vary inversely to maternal androgens in Eastern bluebirds: an experimental study. *Funct. Ecol.* 20, 449–456.
- Nephew, B.C., Romero, L.M., 2003. Behavioral, physiological, and endocrine responses of starlings to acute increases in density. *Horm. Behav.* 44, 222–232.
- Nilsson, J.-A., Råberg, L., 2001. The resting metabolic cost of egg laying and nestling feeding in great tits. *Oecologia* 128, 187–192.
- Pereira, R.J.G., Duarte, J.M.B., Negrão, J.A., 2006. Effects of environmental conditions, human activity, reproduction, antler cycle and grouping on fecal glucocorticoids of free-ranging Pampas deer stags (*Ozotoceros bezoarticus bezoarticus*). *Horm. Behav.* 49, 114–122.
- Pike, T.W., Petrie, M., 2003. Potential mechanisms of avian sex manipulation. *Biol. Rev.* 78, 553–574.
- Pike, T.W., Petrie, M., 2005a. Maternal body condition and plasma hormones affect offspring sex ratio in peafowl. *Anim. Behav.* 70, 745–751.
- Pike, T.W., Petrie, M., 2005b. Offspring sex ratio is related to paternal train elaboration and yolk corticosterone in peafowl. *Biol. Lett.* 1, 204–207.
- Pike, T.W., Petrie, M., 2006. Experimental evidence that corticosterone affects offspring sex ratios in quail. *Proc. R. Soc. Lond., B* 273, 1093–1098.
- Pilz, K.M., Smith, H.G., 2004. Egg yolk androgen levels increase with breeding density in the European starling, *Sturnus vulgaris*. *Funct. Ecol.* 18, 58–66.
- Pilz, K.M., Smith, H.G., Sandell, M.I., Schwabl, H., 2003. Interfemale variation in egg yolk androgen allocation in the European starling: do high-quality females invest more? *Anim. Behav.* 65, 841–850.
- Pyle, P., 1997. Identification Guide to North American Birds—Part 1. Slate Creek Press, Bolinas, CA USA. 732 pp.
- Remage-Healey, L., Romero, L.M., 2001. Corticosterone and insulin interact to regulate glucose and triglyceride levels during stress in a bird. *Am. J. Physiol., Regul. Integr. Comp. Physiol.* 281, R994–R1003.
- Romero, L.M., 2004. Physiological stress in ecology: lessons from biomedical research. *Trends Ecol. Evol.* 19, 249–255.
- Romero, L.M., Remage-Healey, L., 2000. Daily and seasonal variation in response to stress in captive starlings (*Sturnus vulgaris*): corticosterone. *Gen. Comp. Endocrinol.* 119, 52–59.
- Romero, L.M., Romero, R.C., 2002. Corticosterone responses in wild birds: the importance of rapid initial sampling. *Condor* 104, 129–135.
- Romero, L.M., Wikelski, M., 2002. Exposure to tourism reduces stress-induced corticosterone levels in Galápagos marine iguanas. *Biol. Conserv.* 108, 371–374.
- Romero, L.M., Reed, J.M., Wingfield, J.C., 2000. Effects of weather on corticosterone responses in wild, free-living passerine birds. *Gen. Comp. Endocrinol.* 118, 113–122.
- Rubolini, D., Romano, M., Boncoraglio, G., Ferrari, R.P., Martinelli, R., Galeotti, P., Fasola, M., Saino, N., 2005. Effects of elevated egg corticosterone levels on behavior, growth, and immunity of yellow-legged gull (*Larus michahellisi*) chicks. *Horm. Behav.* 47, 592–605.
- Saino, N., Romano, M., Ferrari, R.P., Martinelli, R., Möller, A.P., 2005. Stressed mothers lay eggs with high corticosterone levels which produce low-quality offspring. *J. Exp. Zool.* 303, 998–1006.
- Sapolsky, R.M., Romero, L.M., Munck, A.U., 2000. How do glucocorticoids influence stress responses? Integrating permissive, suppressive, stimulatory, and preparative actions. *Endocr. Rev.* 21, 55–89.
- Schwabl, H., 1993. Yolk is a source of maternal testosterone for developing birds. *Proc. Natl. Acad. Sci. U. S. A.* 90, 11446–11450.
- Schwabl, H., 1997. Maternal steroid hormones in the egg. In: Harvey, S., Etches, R.J. (Eds.), *Perspectives in Avian Endocrinology*. Journal of Endocrinology Limited, Bristol, pp. 3–13.
- Seckl, J.R., 2001. Glucocorticoid programming of the fetus; adult phenotypes and molecular mechanisms. *Mol. Cell. Endocrinol.* 185, 61–71.
- Sinervo, B., DeNardo, D.F., 1996. Costs of reproduction in the wild: path analysis of natural selection and experimental tests of causation. *Evolution* 50, 1299–1313.
- Smith, H.G., 2004. Selection for synchronous breeding in the European starling. *Oikos* 105, 301–311.
- Suorsa, P., Helle, H., Huhta, E., Jäntti, A., Nikula, A., Hakkarainen, H., 2003. Forest fragmentation is associated with primary brood sex ratio in the treecreeper (*Certhia familiaris*). *Proc. R. Soc. Lond., B* 270, 2215–2222.
- Uller, T., Olsson, M., 2006. Direct exposure to corticosterone during embryonic development influences behaviour in an ovoviviparous lizard. *Ethology* 112, 390–397.
- Verboven, N., Evans, N.P., D’Alba, L., Nager, R.G., Blount, J.D., Surai, P.F., Monaghan, P., 2005. Intra-specific interactions influence egg composition in the lesser black-backed gull (*Larus fuscus*). *Behav. Ecol. Sociobiol.* 57, 357–365.
- Vézina, F., Williams, T.D., 2002. Metabolic costs of egg production in the European starling (*Sturnus vulgaris*). *Physiol. Biochem. Zool.* 75, 377–385.
- Vézina, F., Salvante, K.G., Williams, T.D., 2003. The metabolic cost of avian egg formation: possible impact of yolk precursor production? *J. Exp. Biol.* 206, 4443–4451.
- Walker, B.G., Boersma, P.D., Wingfield, J.C., 2005. Physiological and behavioral differences in Magellanic penguin chicks in undisturbed and tourist-visited locations of a colony. *Conserv. Biol.* 19, 1571–1577.
- Walker, B.G., Boersma, P.D., Wingfield, J.C., 2006. Habituation of adult Magellanic penguins to human visitation as expressed through behavior and corticosterone secretion. *Conserv. Biol.* 20, 146–154.
- Williams, T.D., 2005. Mechanisms underlying the costs of reproduction. *Bioscience* 55, 39–48.
- Wingfield, J.C., 2005. The concept of allostasis: coping with a capricious environment. *J. Mammal.* 86, 248–254.
- Wingfield, J.C., Bruener, C., Jacobs, J., 1997. Corticosterone and behavioral responses to unpredictable events. In: Harvey, S., Etches, R.J. (Eds.), *Perspectives in Avian Endocrinology*. Journal of Endocrinology Ltd., Bristol, UK, pp. 267–278.
- Wingfield, J.C., Maney, D.L., Breuner, C.W., Jacobs, J.D., Lynn, S., Ramenofsky, M., Richardson, R.D., 1998. Ecological bases of hormone-behavior interactions: the “emergency life history stage”. *Am. Zool.* 38, 191–206.
- Wittingham, L.A., Schwabl, H., 2002. Maternal testosterone in tree swallow eggs varies with female aggression. *Anim. Behav.* 63, 63–67.