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What causes the decrease in haematocrit during egg production?

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Summary

1. Anaemia has been reported in wild animals, typically associated with traumatic events or ill health. However, female birds routinely become 'anaemic' during egg-laying; we sought to determine the causes of this reduction in haematocrit.
2. Haematocrit in female European Starlings (*Sturnus vulgaris* Linnaeus) decreased between pre-breeding and egg-laying in 3 out of 4 years (the decrease was marginally non-significant in the fourth year). This was independent of changes in ambient temperature altering the metabolic requirements for thermoregulation.
3. There was a positive relationship between haematocrit and plasma levels of the yolk precursor vitellogenin among egg-laying birds, supporting the hypothesis that the initial reduction in haematocrit is caused by increased blood volume associated with osmoregulatory adjustments to elevated levels of yolk precursors.
4. However, haematocrit did not always recover upon cessation of egg production, remaining low at clutch completion (2 of 4 years), incubation (1 of 2 years) and chick rearing (1 of 4 years), suggesting an additional cause of the prolonged reduction in haematocrit.
5. Given the magnitude and prolonged nature of the changes in haematocrit we report, and the interannual variation in haematocrit even during chick-rearing (47–54%), we suggest that 'anaemia' associated with egg production might have implications for aerobic performance during later stages of breeding.

Key-words: Aerobic performance, cost of reproduction, egg-laying, hematocrit

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Introduction

Anaemia is a common manifestation of many human pathophysiologicals and can result from trauma (rapid blood loss), deficient erythropoiesis (red blood cell production) or excessive haemolysis associated with tissue dysfunction or nutrient (iron) deficiency (e.g. Beer & Berkow 1999; Goldman & Bennett 2000). In humans, anaemia can be defined as a decrease in haematocrit of two standard deviations from the mean of a 'normal' population (West 1985), and even mild anaemia is considered to be indicative of an underlying disorder (Beer & Berkow 1999). Anaemia has been reported in

free-living birds and mammals, but here it is also typically associated with traumatic events or ill health, e.g. severe dehydration or nutritional stress, toxicity, or high blood loss due to parasites (Franzmann & LeResche 1978; Hoffman *et al.* 1985; Vleck & Priedkalns 1985; Boismenu, Gauthier & Larochelle 1992; Whitworth & Bennett 1992). However, a decrease in haematocrit has also been observed in birds during 'normal' reproduction. Specifically, haematocrit has been found to drop routinely between pre-laying (i.e. arrival on the breeding grounds or nest-building) and egg-laying (Silverin 1981; Jones 1983; Morton 1994).

A number of hypotheses have been put forward to explain this reduction in haematocrit during egg production. Haematocrit may decline because of an increase in blood volume (i.e. haemodilution), the latter being an osmotic adjustment in response to the appearance of substantial levels of yolk precursors in the plasma (Kern, DeGraw & King 1972; Morton 1994; Reynolds & Waldron 1999); for example, plasma vitellogenin levels increase from undetectable (<5 µg/

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Table 1. Summary of predictions of hypotheses to explain the reduction in haematocrit during egg production. – indicates no prediction because plasma vitellogenin will be at basal levels

Hypothesis	Trait	Prediction	
		During egg production	During subsequent stages (i.e. incubation, chick-rearing)
Haemodilution	Haematocrit	Reduced compared with other stages	Same as pre-breeding
	Plasma osmolarity	Same as pre-breeding	Same as pre-breeding
	Correlation between plasma vitellogenin levels and haematocrit	Positive relationship	–
Direct effect of oestradiol on erythropoiesis	Haematocrit	Reduced compared with pre-breeding	Gradual recovery to pre-breeding levels
	Plasma osmolarity	Increased compared with other stages	Lower than during egg production
	Correlation between plasma vitellogenin levels and haematocrit	No relationship or negative relationship	–
Nutritional stress	Haematocrit	Reduced compared with pre-breeding	No recovery or further decline after egg-laying
	Plasma osmolarity	Increased compared with other stages	Lower than during egg production
	Correlation between plasma vitellogenin levels and haematocrit	No relationship	–
	Correlation between haematocrit and body mass	Positive relationship	Positive relationship
Temperature	Haematocrit	Negative correlation with ambient temperature No reduction during laying when statistically controlling for ambient temperature	Negative correlation with ambient temperature

100 ml) to 2.5 g/100 ml at the onset of egg production (Redshaw & Follett 1976), while plasma triglyceride levels increase from 4 mg ml⁻¹ to 24 mg ml⁻¹ owing to secretion of the second triglyceride-rich yolk precursor, VLDL_y (e.g. Christians & Williams 1999). A second possible explanation for the decrease in haematocrit is that elevated oestradiol levels during egg-laying directly reduce the production of red blood cells (Kern *et al.* 1972). High dose oestrogens suppress haematopoiesis in the bones of quail (Clermont & Schrar 1979) and mice (Perry *et al.* 2000), and bone marrow is an important site for erythropoiesis in adult birds (John 1994). Thirdly, erythropoiesis could be reduced due to nutritional stress associated with reproduction (Jones 1983). An additional possibility is that the apparent drop in haematocrit during egg-laying is actually an artefact caused by seasonal changes in temperature; ambient temperatures may be lower prior to egg-laying, requiring a higher pre-laying haematocrit in order to support increased thermoregulation (e.g. Yahav *et al.* 1997).

We sought to distinguish between these competing hypotheses by measuring haematocrit throughout the entire breeding season since previous studies have documented a decrease in haematocrit between pre-breeding and egg-laying only. In addition, correlations between haematocrit and the plasma concentrations of vitellogenin (the primary yolk precursor), plasma osmolarity, ambient temperature and body mass were also investigated.

PREDICTIONS

The following predictions are summarized in Table 1. The hypotheses are not mutually exclusive and therefore a combination of patterns may be observed if more than one factor is important.

Haemodilution

If the drop in haematocrit during egg production were due solely to an increase in plasma volume serving as an osmotic adjustment to elevated levels of yolk precursors (and not a reduction in the number of red blood cells), then haematocrit should return to normal immediately after egg production, when yolk precursor concentrations return to basal levels (Challenger *et al.* 2001). Furthermore, if haemodilution compensates for the presence of precursors in the circulation, then osmolarity should remain constant throughout reproduction. Finally, there should be a positive relationship between haematocrit and the plasma levels of vitellogenin among egg-laying birds, since increased haemodilution will reduce both haematocrit and vitellogenin concentration.

Direct effect of oestradiol on erythropoiesis

If the production of red blood cells is reduced by oestradiol, haematocrit may not recover to pre-laying

levels until some time after egg-laying (see Discussion). In the absence of an increase in plasma volume, osmolarity will increase during egg-laying because of the presence of the yolk precursors. There may be no relationship between plasma vitellogenin levels and haematocrit, or there may be a negative relationship; egg-laying females with relatively high circulating levels of oestradiol may have high plasma vitellogenin and low haematocrit.

Nutritional stress

As with the previous hypothesis, osmolarity is expected to increase during egg production in the absence of an increase in plasma volume. However, if the reduction in haematocrit were due to nutritional stress, it would not necessarily recover after egg-laying and potentially could continue to decrease as incubation and chick rearing are also energetically demanding (Williams & Vézina 2001). A positive relationship between body mass and haematocrit would be expected, since individuals in a better nutritional state would have greater body mass and higher haematocrit.

Artefact of seasonal changes in temperature

If the apparent drop in haematocrit during egg production is entirely due to changes in temperature (i.e. increased ambient temperature during laying), then within stages of breeding there should be a negative correlation between haematocrit and ambient temperature at the time of sampling, and no difference in haematocrit between stages of breeding when ambient temperature is included as a covariate in the analysis.

Materials and methods

Fieldwork was carried out between March and June 1998–2001 on a free-living, nestbox breeding population of European Starlings at the Pacific Agri-Food Research Centre (PARC) in Agassiz, British Columbia (49°14' N, 121°46' W). Pre-breeding females were caught away from their nestboxes using mist nets while they were roosting in barns, and breeding females were caught while roosting in their nestbox at night. In most years females were caught and blood sampled at five stages of the reproductive cycle (each female was sampled at one stage only): (a) pre-breeding, March (1998, $n = 8$; 1999, $n = 19$; 2000, $n = 19$; 2001, $n = 17$); these birds had not begun nest building and showed no signs of follicle development; (b) egg-laying, April ($n = 14$, 34, 20 and 14, respectively); all birds were sampled on the night that they laid their first egg; (c) clutch completion ($n = 16$, 11, 5 and 15, respectively), females caught 2 days after laying their last egg; (d) mid-incubation, 4–8 days after clutch completion ($n = 32$, 0, 5, 0, respectively); and (e) chick-rearing, 5–15 days after hatching ($n = 21$, 20, 12, 20, respectively). In addition, in 1999 further birds ($n = 75$) were blood sampled during the

laying cycle on the night that they laid their first to fifth egg (see Vézina & Williams 2002 for further details). We did not sample males because it is virtually impossible to catch males, especially during the egg-laying and clutch completion stages of breeding. Furthermore, males would not necessarily provide a useful 'baseline' as they may exhibit their own pattern related to the reproductive cycle.

All birds were blood sampled (800 μ l) from the brachial vein between 22:00 and 01:00 Pacific Standard Time (PST), haematocrit was measured using electronic callipers (± 0.01 mm) and determined as percentage of packed red cell volume to total column height (plasma plus packed red cell volume). All haematocrit measurements were made within 3 h of blood sampling. In 1999–2001 some birds were sampled up to 5 h after capture following respirometry measurements for a separate experiment (see Vézina & Williams 2002). However, there was no significant difference in haematocrit for birds sampled after this procedure compared with those sampled soon after capture ($P > 0.45$). Plasma osmolarity was measured on plasma samples in 1999 for birds at different stages of egg-laying, and for a further set of plasma samples obtained in 2002 from birds at the 1-egg stage of laying ($n = 8$), incubation ($n = 8$) and chick-rearing ($n = 8$). Osmolarity was measured in triplicate by vapour pressure osmometry using a Wescor B 5500 (CV of repeated measurements = 0.8%, $n = 86$). Plasma vitellogenin was assayed in laying birds using the vitellogenin zinc method of Mitchell & Carlisle (1991) validated for passerines (see e.g. Williams & Martyniuk 1999; Challenger *et al.* 2001). Inter- and intra-assay coefficients of variation were 6.2% and 5.3%, respectively. Plasma vitellogenin was not assayed in 1998. Temperature data were obtained from the Environment Canada weather station at Agassiz (<http://climate.weatheroffice.ec.gc.ca>). All procedures were carried out in accordance with the guidelines of the Canadian Committee on Animal Care (Simon Fraser University permit no. 442B).

Results

Haematocrit was independent of sampling time and the delay between capture and blood sampling ($P > 0.15$ in all cases). Similarly, neither of these factors was found to significantly affect vitellogenin levels (see Challenger *et al.* 2001), therefore we did not control for these variables in subsequent analyses.

TEMPERATURE

To investigate the possibility that the drop in haematocrit during egg-laying was an artefact of seasonal changes in ambient temperature, we examined the correlation between haematocrit and temperature on the day of sampling, within each stage of breeding. For each breeding stage, a general linear model was performed with haematocrit as the dependent variable

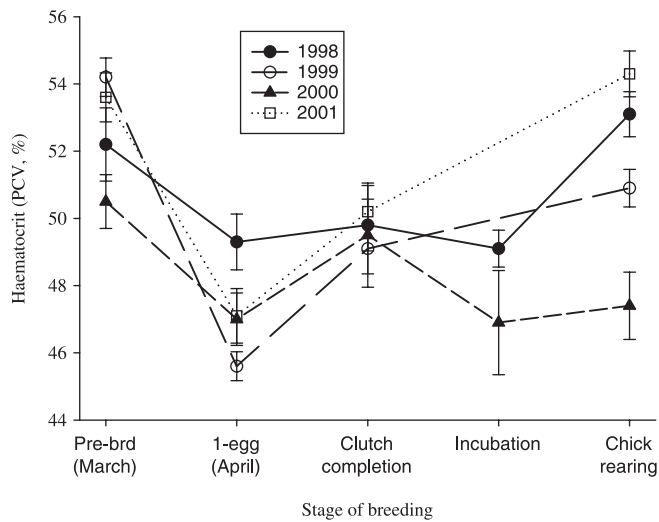


Fig. 1. Variation in haematocrit (PCV, %) of female European starlings in relation to breeding stage and year. Values are means \pm 1 SE.

and temperature, year and the temperature * year interaction as independent terms in the model. The temperature * year interaction term was not significant for any stage and therefore this term was removed from the model. Haematocrit was not correlated with temperature within any stage ($P > 0.2$ in all cases), regardless of whether year was entered in the model or not. Furthermore, the variation in temperature between stages generally did not match the variation in haematocrit (data not shown). While temperature does not appear to influence haematocrit over the range of temperatures observed in this study, we included temperature as a covariate when investigating changes in haematocrit with breeding stage (see below).

STAGE OF BREEDING

The relationship between haematocrit and stage of breeding varied among years (Fig. 1; interaction term, stage * year, $F_{10,301} = 4.22$, $P < 0.001$) so we analysed data for each year separately. Haematocrit varied with breeding stage in all 4 years (1998, $F_{4,90} = 6.78$, $P < 0.001$; 1999, $F_{3,83} = 52.4$, $P < 0.001$; 2000, $F_{4,60} = 3.11$, $P < 0.025$; 2001, $F_{3,65} = 19.4$, $P < 0.001$; Fig. 1). Temperature was not a significant covariate in either the

multiyear analysis or the analyses by year ($P > 0.7$ in all cases) and was therefore removed from the model.

Comparisons of mean haematocrit between each stage and pre-breeding haematocrit are presented in Table 2. Haematocrit decreased significantly during egg-laying in 3 out of 4 years. Furthermore, there was evidence for reduced haematocrit (compared with pre-breeding levels) at clutch completion in 2 out of 4 years, during incubation in 1 out of 2 years and during chick-rearing in 1 out of 4 years. In 1999 and 2001 mean haematocrit during laying was >2 standard deviations lower than pre-breeding values (i.e. mean haematocrit was typical of an 'anaemic' condition). Using the same 2 standard deviations criterion, 15–78% and 36–100% of birds were anaemic during egg-laying and at clutch completion, respectively, in the 4 years of study, 14/32 birds (44%) were 'anaemic' during incubation in 1998, and 4/20 birds (20%) were 'anaemic' during chick-rearing in 1999.

PLASMA OSMOLARITY

In 1999, plasma osmolality did not vary throughout egg-laying ($P > 0.90$ for all contrasts) but increased significantly at clutch completion, 2 days after the last egg was laid (Fig. 2a; contrast of mean for 6th-egg vs clutch completion, $P < 0.01$; ANOVA for overall analysis, $F_{6,81} = 10.38$, $P < 0.001$). We confirmed the lower plasma osmolality during laying by comparing 1-egg birds with incubating and chick-rearing birds in 2002 (Fig. 2b). Osmolality varied with breeding stage ($F_{3,23} = 9.46$, $P < 0.001$) and was lower in 1-egg birds compared with both incubating ($P < 0.01$) and chick-rearing birds ($P < 0.025$). Osmolality of incubating and chick-rearing birds did not differ ($P > 0.90$) and mean osmolality for these breeding stages in 2002 was very similar to that for clutch completion birds in 1999 (321–323 mmol kg^{-1} vs 324 mmol kg^{-1}).

PLASMA VITELLOGENIN LEVELS

To investigate whether the reduction in haematocrit was due to haemodilution associated with increased blood volume, we examined the relationship between haematocrit and plasma vitellogenin levels within

Table 2. Comparison of mean haematocrit at each stage of breeding and mean pre-breeding levels; reduction = (mean pre-breeding haematocrit – mean breeding haematocrit) * 100/mean pre-breeding haematocrit, therefore negative values indicate that haematocrit was higher during breeding than prior to breeding. Unadjusted P -values are presented for each comparison (i.e. breeding vs pre-breeding) and so to maintain $\alpha = 0.05$ within years, P -values must be below 0.013 (i.e. 0.05/4) in 1998 and 2000 and below 0.017 (i.e. 0.05/3) in 1999 and 2001 to be judged significant (P -values highlighted in bold). – indicates that no females were sampled

Stage of breeding	1998		1999		2000		2001	
	Reduction (%)	P -value	Reduction (%)	P -value	Reduction (%)	P -value	Reduction (%)	P -value
1-egg	5.7	0.03	15.8	<0.001	6.8	0.003	12.1	<0.001
Clutch completion	4.6	0.07	9.5	<0.001	1.9	0.589	6.3	0.002
Incubation	6.1	0.01	–	–	7.1	0.044	–	–
Chick rearing	–1.6	0.51	6.0	<0.001	6.2	0.018	–1.5	0.438

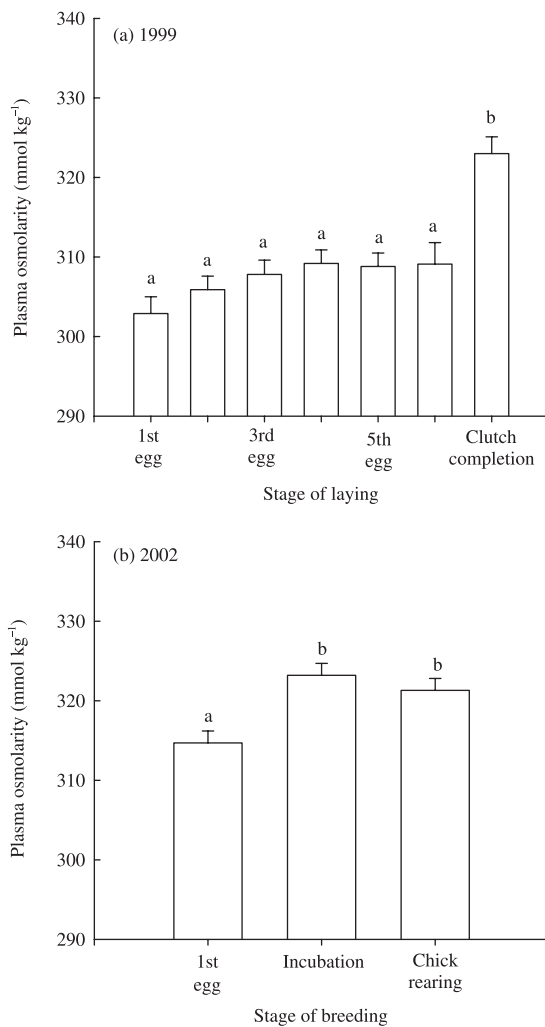


Fig. 2. Variation in plasma osmolarity (mmol kg⁻¹) in female European starlings in relation to (a) stage of egg-laying in 1999, and (b) stage of breeding in 2002. Values are means ± 1 SE, and columns sharing the same letter are not significantly different.

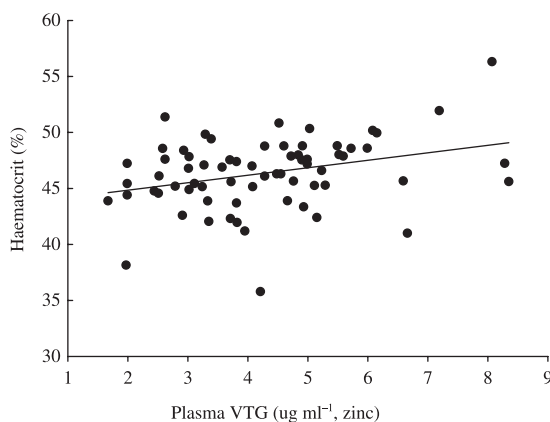


Fig. 3. Relationship between haematocrit and plasma vitellogenin in female European Starlings at the 1-egg stage of laying.

($P > 0.4$). Without the interaction term in the model, there was a significant relationship between haematocrit and vitellogenin ($F_{1,67} = 4.4$; $P = 0.04$) but there was no significant variation between years ($F_{2,67} = 0.4$; $P > 0.6$). Pooling all years, the correlation between haematocrit and vitellogenin was positive and highly significant ($r = 0.32$; $P < 0.01$; Fig. 3).

BODY MASS

To test whether birds in a poorer nutritional state had lower haematocrit, we examined the relationship between body mass and haematocrit within each breeding stage. In a general linear model with haematocrit as the dependent variable and body mass, year and the body mass * year interaction as independent terms, the interaction term was not significant for any stage ($P > 0.3$ for all stages except clutch completion, for which $P > 0.1$). After the interaction term was removed from the model, haematocrit was negatively correlated with body mass only during laying ($F_{1,77} = 4.4$; $P = 0.04$; $r = -0.23$), with year included in the model ($F_{3,77} = 5.8$; $P < 0.01$). The relationship between haematocrit and body mass was not significant at any other stage of breeding ($P > 0.05$ in all cases).

Discussion

Female European Starlings showed a significant decrease in haematocrit associated with egg production in 3 out of 4 years in our study (the decrease was marginally non-significant in the fourth year). Previous studies have similarly reported decreases in haematocrit between arrival, nest-building or pre-laying birds and those producing eggs, e.g. 56% to 45% in *Quelea quelea* (Jones 1983), 56% to 51% in *Zonotrichia leucophrys* (Morton 1994) and 51% to 42% in *Ficedula hypoleuca* (Silverin 1981). We investigated the causes of this decrease in haematocrit, testing a number of competing hypotheses, and ruled out the possibility that the change in haematocrit in this system was caused by seasonal variation in ambient temperature.

The positive relationship between haematocrit and plasma levels of vitellogenin provided support for the haemodilution hypothesis, i.e. that the initial reduction in haematocrit is caused by an increase in blood volume associated with osmoregulatory adjustments to elevated levels of yolk precursors and other blood constituents (Kern *et al.* 1972; Verrinder Gibbins & Robinson 1982; Reynolds & Waldron 1999). This is perhaps not surprising given that egg production probably induces the most marked perturbation in plasma composition ever experienced by healthy adult birds (see Introduction).

However, it is clear that haemodilution in response to the presence of yolk precursors is not the only factor involved because haematocrit did not always return to pre-breeding levels at clutch completion, even though yolk precursor levels have decreased to zero by this

egg-laying birds. In a general linear model with haematocrit as the dependent variable and vitellogenin, year and the vitellogenin * year interaction term as independent terms, the interaction term was not significant

stage (Challenger *et al.* 2001). Low haematocrit persisted in some years through incubation and/or chick-rearing and, in one year, 20% of chick-rearing birds were 'anaemic' (based on haematocrit values more than 2 standard deviations lower than pre-breeding values). Furthermore, mean haematocrit varied markedly from 47 to 54% among chick-rearing birds in different years (Fig. 1). Either a direct suppression of erythropoiesis by elevated oestradiol levels during egg production or nutritional stress could explain the prolonged depression of haematocrit. Although our data do not allow us definitively to distinguish between these two hypotheses, we did not find any evidence of the latter: there was no positive relationship between haematocrit and body mass at any stage of breeding, and haematocrit did not continue to decline after laying in any year (see Table 1 and Predictions).

Estimates of the life span of avian red blood cells (30–42 day; Rodnan, Ebaugh & Spivey Fox 1956) suggest that a transient inhibition of erythropoiesis could induce a prolonged reduction in haematocrit. Kendall & Ward (1974) reported an increase in thymus gland size during incubation and chick-rearing which they argued facilitated a recovery of haematocrit through increased erythropoiesis (Fonfria *et al.* 1983; in contrast to mammals, the avian spleen does not act as a storage site for erythrocytes, John 1994). An alternative that we have not yet discussed is that the changes in haematocrit were due to changes in the size of the red blood cells. However, avian erythrocytes are known to be able to maintain volume homeostasis with hypo-osmotic changes many-fold greater than that seen in our study (Kregenow 1971; but see Davey, Lill & Baldwin 2000). Furthermore, while oestrogen treatment in Japanese Quail resulted in changes in blood volume and erythrocyte mass, the latter were negligible (Nirmalan & Robinson 1973; Verrinder Gibbins & Robinson 1982).

The reduction in osmolarity during laying compared with subsequent stages and the negative relationship between haematocrit and body mass during laying were unexpected observations (see Table 1 and Predictions). The decrease in plasma osmolarity during laying might be functional, perhaps creating a favourable osmotic gradient to facilitate transfer of water into the egg. If this is the case, however, it is not a universal requirement for egg formation since in Zebra Finches (*Taeniopygia guttata*) there is a similar decrease in haematocrit during laying but no change in plasma osmolarity (T. D. Williams, unpublished data). Body mass during laying is confounded with the substantial mass of the reproductive organs (i.e. the enlarged ovary and oviduct) as well as the oviducal egg, making the negative relationship with haematocrit difficult to interpret.

Whatever the cause of the prolonged decrease in haematocrit, it could have important consequences for the female: haematocrit provides a measure of oxygen carrying capacity and low haematocrit might result in a reduction in aerobic performance. Several studies

have shown a positive relationship between haematocrit and aerobic performance (Hammond *et al.* 2000) or flight performance (e.g. Carpenter 1975; Saino *et al.* 1997) in birds, while other studies have reported a positive correlation between haematocrit and reproductive performance (Dufva 1996; Horak, Ots & Murumagi 1998). The magnitude of the variation observed in our study between breeding stages and between years in chick-rearing birds is as great or greater than increases in haematocrit in response to elevated metabolic demand (e.g. thermoregulation, Swanson 1990; altitude, Clemens 1990; migration, Piersma, Everaarts & Jukema 1996). Given the energetic demands of chick-rearing (Williams & Vézina 2001), a prolonged reduction in haematocrit could have substantial consequences for a female's ability to feed chicks and avoid predators, thus providing a potential mechanism for 'costs of reproduction' (*sensu* Monaghan & Nager 1997; Monaghan, Nager & Houston 1998).

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