

Intraspecific variation in reproductive physiology and egg quality in the European Starling *Sturnus vulgaris*

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Egg mass shows large intraspecific variation in birds and is repeatable within individuals. The mechanisms underlying this variation are unknown. We hypothesized that measures of egg quality (the mass of yolk protein, yolk lipid, and albumen protein) would be positively correlated with the plasma pools of the yolk precursor vitellogenin, and the masses of the oviduct, metabolic machinery (liver, heart, lungs, kidneys, gizzard, small intestine and pancreas), and endogenous stores of protein and lipid. We tested these predictions in European Starlings *Sturnus vulgaris* collected at the peak of egg production effort. In contrast to our predictions, both yolk protein and yolk lipid were negatively correlated with plasma vitellogenin levels. Albumen protein was positively related to oviduct mass, but other aspects of body composition failed to explain variation in egg quality. Hence, while we observed correlations between egg composition and peripheral systems (circulating precursor pools and the oviduct), we found no evidence that egg quality is determined by more general processes, i.e., the supply and processing of nutrients.

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Intraspecific variation in performance provides a valuable resource for studying the evolution of phenotypes and their underlying physiology (Garland 1984, Bennett 1987). Propagule size is an important and well-studied life-history parameter, and yet intraspecific variation in this trait is not understood (Bernardo 1996). For example, even though avian eggs supply all of the nutrients required for the pre-hatching development of the embryo (Burley and Vadehra 1989), the size of this nutrient supply (i.e., egg mass) shows large variation within species (Cooke et al. 1995, Williams 1996a). This variation may have consequences for offspring survival during the first days after hatching (Williams 1994) and perhaps longer (Hipfner and Gaston 1999, Styrsky et al. 1999).

Egg mass is highly repeatable within individual birds between breeding seasons (Lessells et al. 1989, Wiggins 1990, Potti 1993, Smith et al. 1993, Flint and Grand 1996), although in some species there is regular and substantial variation within clutches (St. Clair 1996, Royle et al. 1999). The mechanistic basis of differences

between individuals is not known (Cooke et al. 1995, Carey 1996, Williams 1996a). Female size, mass and mass-derived measures of condition (e.g., residuals from a regression of mass on size) often explain little (< 15%) of the variation in egg mass among individuals in a variety of avian groups from passerines (e.g., Wiggins 1990, Williams 1996a) to waterfowl (e.g., Cooke et al. 1995, but see Hepp et al. 1987). Similarly, nutrition has a relatively small (ca. 12%) effect on egg mass (Selman and Houston 1996, Williams 1996b, Ramsay and Houston 1998) compared to the large intraspecific variation in this trait (typically 40%–50% difference between smallest and largest egg mass; Wiggins 1990, Smith et al. 1993, Cooke et al. 1995, Perrins 1996, Williams 1996a). Furthermore, supplementation with a high-protein or high-lipid diet does not diminish the magnitude of variation (Williams 1996b).

The mechanisms which determine egg size remain unknown even though the physiological systems involved in egg production have been studied extensively in poultry (Etches 1996). The growth of the follicles

(yolks) in the ovary is known to be supported by the liver's production of two main yolk precursors, vitellogenin and yolk-targeted very-low density lipoprotein (VLDL) (Wallace 1985). Vitellogenin and VLDL, the primary sources of yolk protein and lipid, respectively, are taken up from circulation by the growing follicles of the ovary via receptor-mediated endocytosis (Stifani et al. 1990). Fully-grown follicles are ovulated and pass through the oviduct where the albumen and shell are deposited (Williams 1998).

The goal of this study was to investigate the physiological mechanisms underlying intraspecific variation in egg mass. We took a correlational approach (Garland 1984, Bennett 1987) to identify potential sources of variation and so provide direction for further experimental work. Because eggs are complex structures, we dissected egg mass into its components: yolk protein, lipid, albumen protein and shell. We hypothesized that (1) the mass of yolk protein and lipid would be positively correlated with the plasma levels of the yolk precursor vitellogenin, i.e., the circulating pool (see Tyler et al. 1990); (2) yolk mass would be positively correlated with liver mass; (3) albumen and shell mass would be positively correlated with oviduct mass; (4) the masses of the protein components (yolk protein, dry albumen) and lipid component (yolk lipid) of the egg would be positively correlated with the masses of potential endogenous stores of protein and/or specific amino acids (i.e., the flight-muscles; Murphy 1986, Houston et al. 1995) and lipid, respectively; and (5) the production of larger eggs would require larger internal organs such as heart, lungs, kidneys, gizzard, small intestine and pancreas (see Daan et al. 1990, Hammond and Diamond 1997, Christians and Williams 1999b). These predictions were tested in a free-living population of European Starlings *Sturnus vulgaris*. In addition, we also collected data on the between-year repeatability of egg size in this population.

Material and methods

General

Data were collected during the breeding seasons of 1996, 1997 and 1998 at the Pacific Agri-food Research Centre (PARC) in Agassiz, British Columbia (49°14' N, 121°46' W), following the guidelines of the Canadian Committee on Animal Care (Simon Fraser University Animal Care Committee Project #442B; PARC ACC Experiment #9702).

Animal collection

In 1996 and 1997, female European Starlings and their eggs were collected from nest-boxes the night after the

first egg of the clutch had been laid, between 21:30 and 03:00 (N = 13 in 1996; N = 25 in 1997). These individuals are the 'one-egg' birds described by Christians and Williams (1999b). Females were collected at the 'one-egg' stage since various models (e.g., Ojanen 1983, Houston et al. 1995, Williams and Ternan 1999) indicate that this is the period of maximum nutritional and energetic investment in eggs. Birds were killed by exsanguination under anesthesia (mixture of ketamine and xylazine at doses of 20 mg/kg and 4 mg/kg, respectively) within 20 min of capture and then frozen.

Body composition

Tarsus, keel and coracoid were measured (± 0.1 mm) at the time of dissection. The following tissues were dissected from the carcass: the left and right *pectoralis* and *supracoracoideus* muscles (hereafter flight-muscles), liver, kidneys, heart, lungs, gizzard, small intestine, pancreas, oviduct (the oviducal egg was removed before weighing) and ovary. The rest of the carcass was autoclaved for nine hours to facilitate homogenization, homogenized and a subsample of the homogenate was used for subsequent analyses. The dry lean masses of the organs and of the carcass were obtained by drying to constant mass (either in a drying oven at 60°C or in a freeze drier) followed by Soxhlet extraction for 8 h, with petroleum ether as the solvent (Dobush et al. 1985). Total body fat was calculated from the difference between the dry mass and the dry lean mass of the organs and carcass. Wet masses are reported for the yolk follicles of the ovary.

Glycogen can make up a substantial proportion of the liver's mass (12%–28% of the dry weight; Rosebrough et al. 1982). Therefore, to obtain the mass of metabolically active hepatic tissue, we determined the dry lean glycogen-free mass of the liver. Liver glycogen was determined using a modification of the method described by Keppler and Decker (1984) using a subsample of liver (ca. 40 mg) that had been frozen in liquid nitrogen immediately after collection. Further details on the measurement of liver glycogen are provided by Christians and Williams (1999b).

Measurement of yolk precursors

Plasma levels of vitellogenin were measured using the vitellogenic zinc method developed for the domestic hen (Mitchell and Carlisle 1991) and validated for passerines (Williams and Martyniuk 2000). This method separates zinc bound to serum albumin from that bound to vitellogenin (i.e., vitellogenic zinc). The concentration of the latter is proportional to the plasma concentration of vitellogenin (Mitchell and Carlisle 1991). The concentration of vitellogenic zinc is obtained from the

difference between zinc in unmanipulated plasma (zinc – Wako Chemicals), and that in plasma that has been depleted of vitellogenin and VLDL by precipitation with dextran sulfate; only 2% of plasma zinc is complexed with VLDL (Mitchell and Carlisle 1991). The inter-assay coefficient of variation for the vitellogenic zinc assays performed in this study was 11.2% (N = 20).

Egg composition

Within 24 h of laying, the eggs of collected females were weighed, boiled for 10 min and then frozen. Eggs were later separated into shell, albumen and yolk. All components were dried and lipids were extracted from the yolks, as described above. Dry albumen and lean dry yolk are approximately 88% protein (Burley and Vadehra 1989), and were used as measures of albumen and yolk protein, respectively (see also Rohwer 1986, Sotherland and Rahn 1987).

Between-year repeatability of mean egg mass

Throughout the three breeding seasons, a larger sample of unmanipulated birds was also studied. For these birds, fresh egg mass was calculated from egg dimensions using an empirical formula ($\text{mass} = 0.0009159 \times \text{length}^{0.954} \times \text{breadth}^{1.877}$; $r^2 = 0.98$, $N = 175$). Females were banded with an aluminium U.S. Fish and Wildlife Service band to permit identification in subsequent years. The between-year repeatability of mean egg mass was calculated for females which were located in more than one year (Lessells and Boag 1987).

Statistical analyses

The lengths of the tarsus, keel, and coracoid were combined into a single index of body size using the first principal component of a principal components analysis (proc PRINCOMP, SAS Institute 1988; Freeman and Jackson 1990). Preliminary analyses indicated that variation in the egg components was not significantly correlated with female size or mass ($|r| < 0.3$, $P > 0.05$ in all cases), or with the date of the first egg ($|r| < 0.2$, $P > 0.1$ in all cases). Therefore, statistically controlling for these

factors was not necessary. All analyses were performed including year and interaction terms as covariates in a general linear model (proc GLM, SAS Institute 1988). In no case were the year or interaction terms considered significant ($\alpha = 0.05$ for year effects; $\alpha = 0.15$ for interactions), indicating that data could be pooled between years. The statistical power to detect interactions is much less than that to detect main effects, and so we used an α of 0.15 to increase our power to detect interactions. This decreased the risk of ignoring real interactions when pooling data.

Except where noted, product-moment correlation coefficients are reported (proc CORR, SAS Institute 1988). To control the type-I error rate, we applied a sequential Bonferroni correction (Rice 1989) when evaluating the significance of correlations. We classified correlations into six groups of tests: one for each of the five hypotheses outlined in the introduction, and a sixth for correlations among egg mass, egg components and follicle masses. An overall α -level of 0.05 was maintained for each group (see Rice 1989). Sample sizes vary slightly because some ovarian follicles burst during dissection, and because some samples were accidentally destroyed during the drying and lipid extraction of egg components.

Results

Variation in egg mass and its components

It has been suggested that variation in fresh egg mass does not necessarily reflect variation in the macronutrient (i.e., protein and lipid) content of eggs (Bernardo 1996). However, in this study fresh egg mass and its components (yolk lipid, yolk protein, albumen protein, and dry shell mass) showed variation of similar magnitude (Table 1). Fifth and 95th percentiles are presented to demonstrate that the large variation is not generated by extreme values.

Fresh egg mass was correlated with the masses of yolk lipid ($r_{37} = 0.61$, $P < 0.0001$), protein ($r_{37} = 0.71$, $P < 0.0001$), albumen protein ($r_{36} = 0.92$, $P < 0.0001$), and shell ($r_{38} = 0.68$, $P < 0.0001$), and all egg components were positively correlated with each other; correlation coefficients ranged from 0.46 to 0.79 ($P < 0.01$ in all cases).

Table 1. Descriptive statistics for fresh egg mass and egg components (excluding any eggs for which the mass of one or more components was missing; N = 35).

| Component | Mean | Coefficient of variation (%) | 5th percentile | 95th percentile |
|----------------------|------|------------------------------|----------------|-----------------|
| Fresh egg (g) | 7.12 | 9.0 | 5.77 | 8.14 |
| Yolk lipid (mg) | 308 | 11.2 | 240 | 357 |
| Yolk protein (mg) | 196 | 11.9 | 153 | 229 |
| Albumen protein (mg) | 542 | 12.6 | 426 | 632 |
| Dry shell (mg) | 435 | 7.9 | 379 | 490 |

Both yolk lipid and yolk protein in the first egg of the clutch were highly correlated with the wet mass of the largest yolk follicle in the ovary ($r_{32} = 0.72$ and $r_{32} = 0.79$, respectively, $P < 0.0001$ in both cases), which would have formed the yolk of the egg laid two days after the first egg (the oviducal egg would have been laid the day after the first). The relationships between yolk lipid and protein and follicle mass were weaker but still significant for the second ($r_{32} = 0.59$ and $r_{32} = 0.61$, respectively, $P < 0.001$ in both cases) and third largest follicles ($r_{31} = 0.46$ and $r_{31} = 0.52$, respectively, $P < 0.01$ in both cases).

All of the correlations among egg mass, egg components and follicle masses described above were considered significant when a sequential Bonferroni correction (Rice 1989) was applied to maintain an overall α -level of 0.05. There were 22 comparisons (four between fresh egg mass and egg components, 12 among egg components, and six among ovarian follicles and yolk lipid and protein) and so the strongest correlation was considered significant at $\alpha = 0.05/22 = 0.0023$, the next strongest correlation was considered significant at $\alpha = 0.05/21 = 0.0024$ and so on.

The correlations between yolk masses and the masses of the ovarian follicles indicate that yolk mass was repeatable for individual females within clutches. Furthermore, among unmanipulated birds, mean egg mass was highly repeatable in individual females laying in different years (repeatability = 0.76, $N = 46$ birds, $P < 0.0001$).

Yolk components and plasma vitellogenin levels

Plasma vitellogenin was negatively correlated with both yolk protein ($r_{36} = -0.51$, $P < 0.002$; Fig. 1a) and yolk lipid ($r_{36} = -0.62$, $P < 0.0001$; Fig. 1b). These correlations were significant using a Bonferroni corrected α -value of $0.05/2 = 0.025$. The variation in the plasma levels of vitellogenin was independent of the time of night that the birds were collected (data not shown).

Egg components and organ masses

The relationships between liver mass and the yolk components were not significant (yolk lipid: $F_{1,31} = 0.24$, yolk protein: $F_{1,31} = 0.33$, $P > 0.5$ in both cases) when controlling for plasma vitellogenin in a general linear model. Albumen protein was correlated with oviduct mass ($r_{36} = 0.46$, $P < 0.01$; Fig. 2), but dry shell mass was not ($r_{38} = 0.19$, $P > 0.1$). The protein components of the egg were not correlated with flight-muscle mass (yolk protein: $r_{37} = 0.06$, $P > 0.5$; albumen protein: $r_{36} = 0.03$, $P > 0.5$). Similarly, yolk lipid was not correlated with total body fat ($r_{37} = 0.17$, $P > 0.3$). Fresh egg mass was also independent of flight-muscle

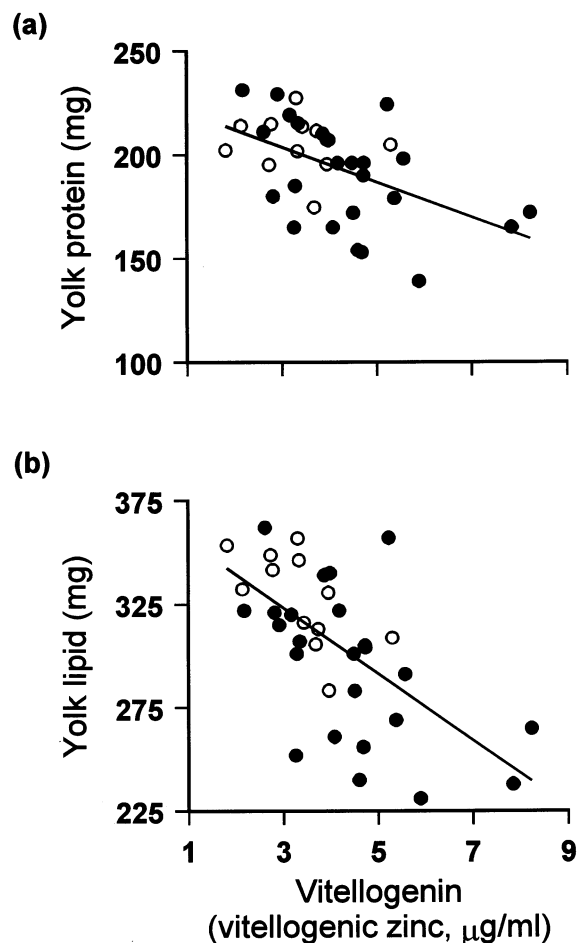


Fig. 1. Relationships between the plasma concentration of vitellogenin and (a) yolk protein; (b) yolk lipid. Open circles represent 1996 data; closed circles represent 1997 data. Regression lines were calculated by pooling data from both years.

mass and total body fat ($P > 0.3$ in both cases). Fresh egg mass and its components were not related to measures of flight-muscle mass standardized for body size, i.e., the residuals of least squares regression of muscle mass on female size ($P > 0.2$ in all cases) (cf. Murphy 1986).

No component of the metabolic machinery involved in digestion (gizzard, small intestine or pancreas) or the processing of energy and nutrients (heart, kidneys, lungs) was correlated with fresh egg mass ($|r| < 0.25$, $P > 0.1$ in all cases) or with the masses of the reproductive tissues (ovary and oviduct; $|r| < 0.35$, $P > 0.05$ in all cases).

Discussion

Previous studies have examined the mechanistic basis of intraspecific variation in important performance traits

such as locomotory performance (Garland 1984, Garland and Else 1987, Friedman et al. 1992), maximum oxygen consumption (Garland 1984), and basal metabolism (Burness et al. 1998). Avian egg size is a performance trait which shows high, repeatable variation with likely consequences for fitness, and yet almost nothing is known about the causes of this variation. Our goal was to identify the physiological mechanisms which determine egg size.

Our measures of performance consisted of the mass and macronutrient composition of the first-laid egg of each female. The high repeatability of egg mass and the close correlations between yolk lipid and protein and the masses of the ovarian follicles (i.e., the yolks of eggs which would have been laid in subsequent days) confirm that the one egg we obtained from each female was representative of the eggs that she would have produced. Our repeatability value for egg mass was identical to that obtained by Smith et al. (1993) for European Starlings. Similarly, Ojanen et al. (1981) found that ca. 84% of variation in egg volume was due to differences between clutches in this species, consistent with general trends observed in most birds (Carey 1996).

Previous studies of intraspecific variation in propagule size have frequently assumed that variation in size reflects variation in egg quality, but as Bernardo (1996) pointed out, this assumption is not necessarily valid. In the present study, however, macronutrient measures of egg quality (yolk lipid, protein, albumen protein and shell mass) did show variation as high as that observed in fresh egg mass. Furthermore, the masses of these components were highly correlated with egg mass, in contrast to Ricklefs' (1977, 1984) observations of weak or non-existent correlations between yolk content and egg mass.

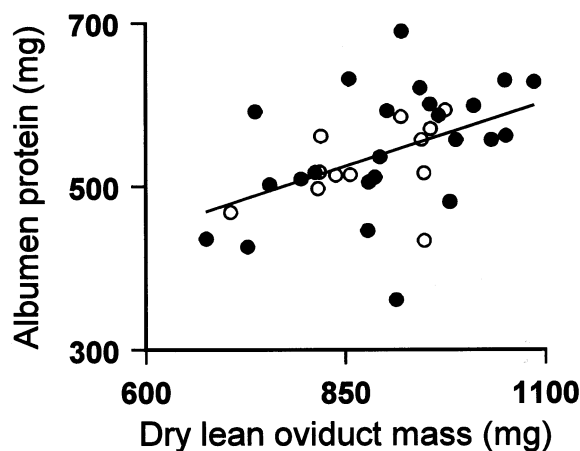


Fig. 2. Relationship between albumen protein and dry lean oviduct mass. Symbols as in Fig. 1.

Yolk components and plasma vitellogenin levels

Plasma concentrations of the yolk precursors varied four-fold within our sample of females (Christians and Williams 1999b). The cause of this large variation is not clear; vitellogenin levels are generally not influenced by diet in captive Zebra Finches *Taeniopygia guttata* (T. D. Williams, unpubl. data). Furthermore, there does not appear to be a diurnal pattern in plasma precursor levels in poultry (Redshaw and Follett 1972), and in this study we could not detect any significant effect of time of sampling (between 21:30 and 03:00). We predicted that this large variation in vitellogenin levels would be related to yolk content, specifically that higher circulating concentrations of vitellogenin would result in more rapid follicular growth and hence larger yolks. In contrast, the amounts of yolk protein and lipid were negatively correlated with plasma vitellogenin levels. A possible explanation for this result is that the amount of nutrients in the yolk is determined primarily by the rate of precursor uptake by the growing follicles. High uptake rates may yield large follicles but may also deplete the circulating pool of vitellogenin, or not allow this pool to build up. Uptake rates would be expected to be positively related to the concentration of vitellogenin in the surrounding medium, as observed in rainbow trout *Salmo gairdneri* ovarian follicles cultured *in vitro* (Tyler et al. 1990). However, precursor concentrations above some threshold may be sufficient to saturate the uptake process (Tyler et al. 1990).

Although vitellogenin is the primary source of yolk protein (Wallace 1985), plasma levels of this precursor were negatively correlated with yolk lipid as well as yolk protein. The uptake of lipid and protein may be mechanistically coupled since a single receptor in the oocyte plasma membrane mediates the uptake of both VLDL and vitellogenin in the chicken (Stifani et al. 1990). The lack of a positive correlation between yolk precursor levels and yolk components is consistent with the observation that exogenous estradiol elevated vitellogenin levels in laying European Starlings but did not increase the mass of yolk protein and lipid or egg mass (Christians and Williams 1999a).

Egg components and organ masses

The mass of the oviduct explained approximately 21% of the variation in albumen protein content. Similarly, Ricklefs (1976) found egg mass to be related to oviduct mass in European Starlings. Hence, an aspect of egg quality (i.e., albumen protein) appears to be determined in part by a peripheral organ (*sensu* Hammond and Diamond 1997, Chappell et al. 1999), the oviduct. Interestingly, we found no evidence to support our hypothesis that variation in egg mass or quality could

be explained by variation in the size of the central machinery (*sensu* Hammond and Diamond 1997, Chappell et al. 1999), i.e., organs involved in the production of the yolk precursors (liver) and/or the supply of nutrients for egg formation (heart, lungs, kidneys, gizzard, small intestine and pancreas).

Finally, endogenous stores of protein and lipid were not correlated with egg composition. Our results contrast with those of Murphy (1986), who found that a measure of "protein availability" (flight-muscle mass standardized by size) explained about 21% of the variance in egg mass. Thus, our results suggest that gross body composition does not offer a useful measure of female quality with regard to egg production. Similarly, female mass or size were not good predictors of egg quality, consistent with many previous studies. For example, Smith et al. (1993) found no correlation between egg mass and tarsus length and only a weak ($r = 0.30$) correlation between egg mass and body mass corrected for tarsus length in European Starlings.

Conclusions

Females may attempt to lay the largest eggs they can, or may lay eggs of some optimum size which is smaller than their physiological maximum (Smith and Fretwell 1974). Whatever the case, the factors which determine a given female's maximum/optimum egg size remain unknown. Our finding that plasma vitellogenin levels were negatively correlated with the mass of yolk protein and lipid may indicate that individuals which produce larger yolks have higher rates of yolk precursor uptake by the ovary (with higher uptake rates depleting the circulating pool of vitellogenin). Furthermore, the amount of albumen protein deposited into each egg was found to be correlated with the mass of the oviduct. The results of the present study therefore suggest that the ovarian uptake of yolk precursors and oviduct mass deserve further study as potential mechanisms underlying intraspecific variation in egg quality.

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