Follicular Development and Plasma Yolk Precursor Dynamics through the Laying Cycle in the European Starling (*Sturnus vulgaris*)

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ABSTRACT

We investigated the quantitative matching of plasma yolk precursor supply (the plasma pool) to follicle demand during yolk formation in European starlings (*Sturnus vulgaris*). Plasma concentrations of the two yolk precursors, vitellogenin (VTG) and very low density lipoprotein (VLDL), were only elevated coincident with rapid yolk development (RYD) and matched variation in total yolky follicle mass. VTG and VLDL were low (<0.4 μg/mL and <4.2 mg/mL, respectively) in nonbreeders and prebreeders with no yolky follicles, and at clutch completion. They increased to 4.02 μg/mL and 19.4 mg/mL in birds with a full follicle hierarchy (F_1–F_4), and concentrations then remained high and actually increased up to the point where only a single, yolky (F_1) follicle remained. However, there was some evidence for mismatching of supply and demand: (*a*) precursor concentrations increased throughout the laying cycle even though the number of developing follicles decreased. We suggest that this is because of a requirement to maintain a large precursor pool to maintain high uptake rates; and (*b*) in birds with a full follicle hierarchy, precursor concentrations were negatively correlated with total follicle mass. This suggests that high uptake rates in large follicles can actually deplete circulating precursor concentrations. Plasma concentrations of both yolk precursors increased rapidly in the early morning with (predicted) time after ovulation, consistent with a lack of fine control of precursor concentrations. However, mean plasma VTG concentrations did not differ between morning or evening samples. In contrast, plasma VLDL concentrations were lower in the morning (16.8 mg/mL) than in the evening (22.9 mg/mL). Although there is marked individual variation in plasma VTG and VLDL (four- to eightfold), both precursors were repeatable in the short term (24 h), and plasma VTG was repeatable over a 14-d interval between successive breeding attempts.

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Introduction

Assuming that the functional capacities of any physiological system have costs (e.g., allocation of biosynthetic energy or space), then natural selection should tend to eliminate unused capacities (Diamond and Hammond 1992). Thus, there should be some quantitative match between the amount of the component(s) of a physiological system and the natural load or demand placed on the system. This idea of economy of design was formalised by Taylor and Weibel (1981) and termed "symmorphosis" (see also Weibel et al. 1998) but has subsequently proven to be controversial (e.g., Garland and Huey 1987; Dudley and Gans 1991). However, the general principle of economy of design is logical and intuitively attractive (Lindstedt and Jones 1987), at least qualitatively (Garland and Carter 1994), although exactly how good the match should be is less obvious (e.g., because of maintenenace of safety factors; Alexander 1981; Hammond et al. 1994). Nevertheless, this model provides a useful, and testable, hypothesis for investigating the structure-function relationships of physiological systems (Diamond and Hammond 1992).

Reproduction represents a biological function that is assumed to be costly in terms of survival and/or future fecundity (Clutton-Brock 1991; Stearns 1992), in part because of costs associated with propagule formation (Bernardo 1996). For example, costs of egg production have been demonstrated in terms of a decrease in subsequent reproductive performance (Monaghan and Nager 1997; Monaghan et al. 1998) and suppression of immune function (Oppliger et al. 1996) within breeding attempts. In oviparous vertebrates, yolk formation makes up a large proportion of the energetic cost of egg formation (e.g., Ojanen 1983; Burley and Vadehra 1989; Williams and Ternan 1999), which in passerine birds may itself account for up to 40%–50% of the daily energy budget (Perrins 1996; Meijer and Drent 1999). During egg formation, the avian liver produces two yolk precursors in response to increasing plasma estrogen concentrations: vitellogenin (VTG) and yolk-targeted very low density lipoprotein (VLDLy), which are the primary sources of yolk protein and lipid, respectively (Deeley et al. 1975; Wallace 1985; Walzem et al. 1999). Vitellogenin and VLDLy particles are secreted into the circulation and taken up by developing oocytes of the ovary via receptor-mediated endocytosis (Wallace 1985; Barber et al. 1991). Vitellogenin production alone comprises approximately 50% of the daily hepatic protein synthesis of the laying hen (*Gallus gallus domesticus*) and may triple the amount of protein secreted into the blood (Gruber 1972). VLDLy production also involves changes in the physical structure and biochemical properties of hepatically produced VLDL (Walzem 1996). Thus, there are likely to be energetic, metabolic, and possibly osmoregulatory costs associated with yolk precursor production, which would predict a close matching between the amount of yolk precursors produced by the liver (i.e., supply) and the amount required for ovarian follicular growth (i.e., demand).

In this article, we first present data on the pattern of follicle development in a free-living passerine, the European starling (*Sturnus vulgaris*), including information on the timing of follicular atresia during follicle development. We then compare variation in follicle mass (as an index of demand) with changes in plasma yolk precursor concentrations at three levels: (1) daily variation throughout the laying cycle, (2) variation among individuals, controlling for stage of laying, and (3) diurnal variation in relation to (predicted) time of ovulation. We predicted that plasma concentrations of the yolk precursors (an index of the pool of precursors available to the developing follicles) would be highest on the day(s) of greatest follicular mass and also that it would be highest in individuals with the largest follicle mass, independent of stage of egg production. Finally, we report on repeatability of plasma VTG and VLDL concentrations within individuals within clutches (over 24 h) and between clutches (over several weeks).

Material and Methods

This study was carried out on a free-living population of European starlings at the Pacific Agri-Food Research Centre (PARC) in Agassiz, British Columbia (49°14′N, 121°46′W), in accordance with the guidelines of the Canadian Committee on Animal Care (Simon Fraser University permit 442B, PARC permit 9702). Approximately 130 nest boxes were installed on farm buildings and telephone poles at this site in 1995. Daily nest box checks were performed to determine laying status of females, and all newly laid eggs were marked in permanent ink to indicate oviposition sequence. Starlings typically lay one egg per day (Feare 1984); therefore, only birds with a continuous daily laying pattern were used in the study. Nonbreeding and prebreeding females (see below for definitions) were caught using mist nets while roosting in barns, and all breeding females were caught while roosting in their nest box at night. Females that were not killed were blood sampled (from the brachial vein), weighed (± 1 g), and banded with an aluminium U.S. Fish and Wildlife Service band to permit later identification.

Variation in Follicular Growth and Plasma Yolk Precursor Concentrations throughout the Laying Cycle

A sample of nonbreeding female starlings were caught in March 1997 ($n = 5$; these birds had no yellow, yolky follicles), and prebreeding females ($n = 23$, 14 in 1997 and 9 in 2000) were caught in April before the first egg was laid in the colony. After the onset of laying, female starlings were caught on the night that they laid their first to fifth eggs ($n = 52$, collected in 1999) as well as at clutch completion ($n = 11$; 2 d after the last egg in the clutch was laid). All birds were blood sampled from the brachial vein between 10:00 P.M. and 1:00 A.M. PST (mean sampling time 12:05 A.M. ± 7 min). Females were then killed by exsanguination under anaesthesia (mixture of ketamine and xylazine at doses of 20 mg/kg and 4 mg/kg, respectively) and any large yolky follicles considered to be undergoing rapid yolk development (RYD) were counted, dissected out, measured, and weighed within 30 min of death. Follicles were determined as being in RYD by their size (>2 mm in diameter) and distinctive yellow colour, which was easily distinguishable from nonvitellogenic, white follicles. In any female, only a maximum of four yolky follicles was observed, and these were classified as F_1 to F_4 follicles, with the F_1 being the largest of the yolky follicles (Johnson 1999). Carcasses were then frozen, and the remaining oviduct and ovary were removed and weighed at a later date. Nonreproductive mass of each dissected female was defined as total body mass minus the mass of the ovary and oviduct.

Plasma samples were assayed for yolk precursors using vitellogenin zinc (Zinc kit, Wako Chemicals) and total triglycerides (Triglyceride E kit, Wako Chemicals) as indices of vitellogenin and VLDL, respectively (Mitchell and Carlisle 1991; Christians and Williams 1999*b*; see also Vanderkist et al. 2000). The VTG-Zn and triglyceride assays had an interassay coefficient of variation of 6.2% ($n = 25$) and 4.2% ($n = 10$), respectively, and an intra-assay coefficient of variation of 5.3% and 4.7%, respectively ($n = 10$).

Diurnal Variation and Repeatability of Yolk Precursors

To investigate diurnal variation and short-term repeatability of plasma yolk precursor concentrations, females ($n = 19$, sampled in 1999) were blood sampled between 10:00 P.M. and 12:00 A.M. on the night after they laid their first egg. They were then held in their respective nest boxes overnight (simply by blocking the nest hole) and blood sampled again the following morning approximately 12 h later (10:00 A.M.–12:00 P.M.). As egg laying occurs between 8:00 and 10:00 A.M. in starlings (Feare 1984) and ovulation occurs within 30 min after oviposition (Etches 1996), nighttime and morning blood sampling occurred 12–15 and 0–2 h after ovulation, respectively. To control for any effect of handling or confinement of females in nest boxes, a second group of females ($n = 18$) were blood sampled in the morning only (10:00 A.M.–12:00 P.M.) on the day their second egg was laid. Between-clutch repeatability of yolk precursor concentrations was determined in females that were blood sampled during their first clutch and then again during their replacement clutch laid after desertion or in response to removal of the first clutch (see Christians and Williams 1999*a*).

Statistical Analysis

All statistical analyses were carried out using SAS (SAS Institute 1990). Preliminary analyses showed that total yolky follicle mass (i.e., F_1–F_4), plasma VTG, and plasma VLDL concentrations were independent of nonreproductive body mass ($P > 0.10$ in all cases). Similarly, time between capture and blood sampling had no effect on plasma VTG and VLDL concentrations ($P > 0.10$ in both cases). Therefore, statistically controlling for these factors was not necessary. Variation in the mass of the yolky follicles and in the plasma concentrations of VTG and VLDL was assessed using general linear models (GLM procedure; SAS Institute 1990), and pairwise comparisons between groups were performed using contrasts (Sokal and Rohlf 1995). When assessing changes in follicle mass and the plasma concentrations of VTG and VLDL throughout the laying cycle, only four pairwise comparisons were made (described below) to reduce the number of contrasts; a Bonferroni-adjusted level of significance was used for each comparison (Sokal and Rohlf 1995), that is, 0.05/4 comparisons = 0.0125, to maintain an experimentwise error rate of 0.05. When examining the effects of diurnal variation on the plasma concentrations of the yolk precursors, comparisons between all three groups were made; thus, $\alpha = 0.05/3 = 0.017$. Repeatability of yolk precursors was calculated following Lessells and Boag (1987).

Results

Variation in Follicular Growth and Plasma Yolk Precursor Concentrations throughout the Laying Cycle

As expected, total yolky follicle mass varied markedly throughout the laying cycle, as follicles were recruited into the hierarchy and initiated rapid yolk development ($F_{11,79} = 268.9$, $P < 0.001$; Fig. 1). Total yolky follicle mass increased from 0.067 ± 0.012 g ($n = 6$) when only the first yolky follicle was present to 1.970 ± 0.046 g ($n = 26$) for birds with a complete F_1–F_4 hierarchy and then decreased to 0.940 ± 0.043 g ($n = 9$) when only a single large yolky follicle was present. Plasma VTG and VLDL concentrations varied significantly with stage of follicle development (VTG, $F_{10,77} = 22.41$, $P < 0.001$; VLDL, $F_{10,77} = 13.52$, $P < 0.001$), and both yolk precursors matched changes in follicle mass fairly closely on the broad scale (Fig. 1). Plasma VTG and VLDL were strongly positively correlated

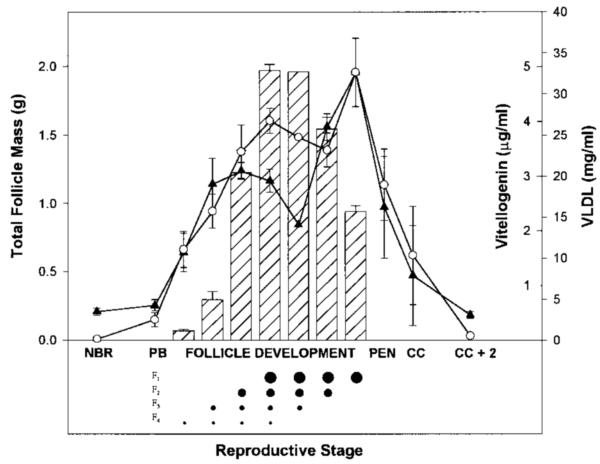

Figure 1. Variation in plasma concentrations of vitellogenin (*open circles*) and very low density lipoprotein (*closed triangles*) in relation to total yolky follicle mass during the laying cycle (*hatched bars*) and pattern of follicle development (*bottom panel*) in female starlings. *NBR* = nonbreeders; *PB* = prebreeders; *PEN* = day of penultimate egg; *CC* = clutch completion; *CC* + 2 = 2 d after clutch completion. Values are means ± 1 SE.

with each other ($F_{1,76} = 78.41$, $P < 0.001$, $r^2 = 0.84$; controlling for stage of follicle development). Both yolk precursors were basal in nonbreeding females (VTG, 0.02 ± 0.02 μg/mL; VLDL, 3.5 ± 0.4 mg/mL) and then increased rapidly to 1.66 ± 0.33 μg/mL and 10.7 ± 2.4 mg/mL, respectively, as soon as a single, small yolky follicle was present ($P < 0.001$ in both cases). When the full follicle hierarchy (F_1–F_4) was established, mean plasma concentrations of VTG and VLDL were 4.01 ± 0.23 μg/mL and 19.4 ± 1.4 mg/mL, respectively (pairwise comparisons with females with single yolky follicle, $P < 0.001$) but varied fourfold between individuals (1.67–6.67 μg/mL and 9.2–39.8 mg/mL respectively). Then, even though total follicle mass declined to the point where the ovary contained only a single F_1 follicle, plasma yolk precursor concentrations remained high and actually continued to increase (VTG, $P = 0.04$; VLDL, $P < 0.001$; pairwise comparison with birds with full hierarchy) reaching a peak on the last day of follicle development (VTG, 4.90 ± 0.62 μg/mL; VLDL, 26.0 ± 1.6 mg/mL; VLDL). Only after follicle development had ceased did the precursor concentrations decrease, returning to basal concentrations 2 d after the cessation of egg laying (concentrations not significantly different from nonbreeding values; $P > 0.90$ in both cases).

In prebreeding females, plasma concentrations of both yolk precursors increased rapidly at the onset of follicle development as the first yolky follicle started to develop (Fig. 2). The relationship between yolk precursor concentrations and follicle mass followed a hyperbolic function (i.e., of the form precursor = [a × follicle mass]/[b + follicle mass]; VTG, $F_{1,26} = 104.4$, $P < 0.001$, $r^2 = 0.80$; VLDL, $F_{1,26} = 102.4$, $P < 0.001$, $r^2 = 0.80$).

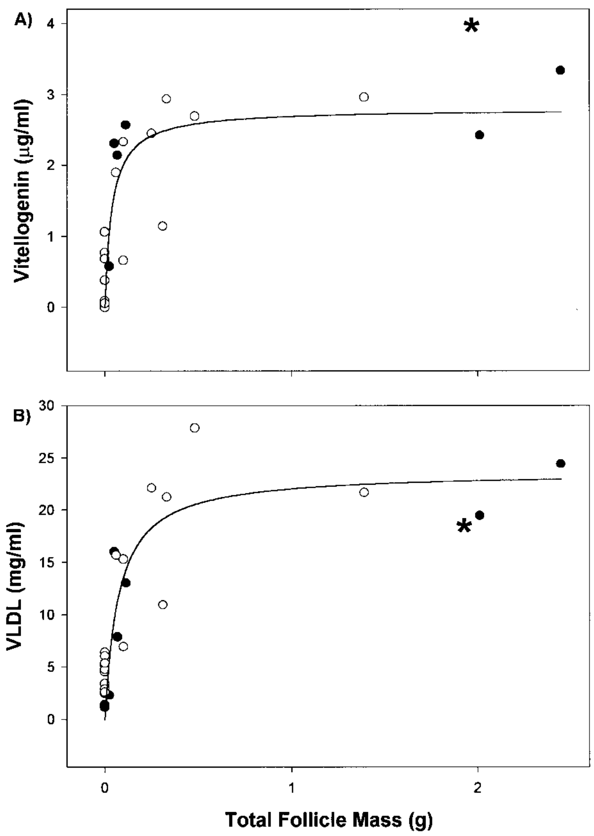

Figure 2. Relationship between total yolky follicle mass and (*A*) plasma vitellogenin and (*B*) plasma very low density lipoprotein (VLDL) concentrations in female starlings before the laying of their first egg. Precursor concentrations remain low until follicles start to develop. *Open circles*, 1997; *filled circles*, 2000. The asterisk indicates mean follicle mass and yolk precursor concentration for birds with a follicle hierarchy (F_1–F_4).

However, once the full follicle hierarchy had been established and during subsequent follicle development (when follicle recruitment had ceased), the relationship between yolk precursor concentrations and yolky follicle mass was reversed, becoming significantly negative (linear regression; VTG, $F_{1,40} = 6.76$, $P < 0.025$, $r^2 = 0.14$; VLDL, $F_{1,40} = 27.57$, $P < 0.001$, $r^2 = 0.40$; Fig. 3). This negative relationship was maintained when stage of follicle development was included as a covariate in a general linear model (VTG, $F_{1,37} = 7.06$, $P < 0.025$; VLDL, $F_{1,37} = 5.23$, $P < 0.05$). Similarly, yolk precursor concentrations and total follicle mass were negatively related, including only birds that had a full follicular hierarchy (VTG, $F_{1,24} = 7.39$, $P < 0.025$, $r^2 = 0.23$; VLDL, $F_{1,24} = 5.22$, $P < 0.05$, $r^2 = 0.17$).

Evidence for Follicular Atresia

Individual follicles progress through the follicular hierarchy on a daily basis; that is, the F_4 follicle on the day the first egg is laid becomes the F_3 follicle on the day the second egg is laid, and so forth. Therefore, we could identify instances of follicular atresia by comparing the frequency of occurrence of developmentally related follicles (i.e., follicles that would give rise to the same egg in a laying sequence) in females collected at two successive egg stages. One-egg females had significantly more F_4 follicles (which would potentially form the sixth egg to be laid) than two-egg females had F_3 follicles (also potential sixth eggs; likelihood ratio chi-square, $\chi^2 = 7.36$, $P < 0.01$; Table 1). Similarly, for follicles that would potentially form the seventh egg in the laying sequence, the frequency of F_3 follicles in three-egg birds was significantly lower than that of F_4 follicles in two-egg birds ($\chi^2 = 7.90$, $P < 0.01$; Table 1). Conversely, there was no difference ($P > 0.05$) in the frequency of F_2 follicles in three-egg birds compared with F_3 in two-egg birds (potential sixth eggs) or in the frequency of F_1 follicles in four-egg birds compared with F_2 follicles in three-egg birds ($P > 0.80$; Table 1). Thus, there was evidence for follicular atresia but only early in follicular development between the F_4 and F_3 stages.

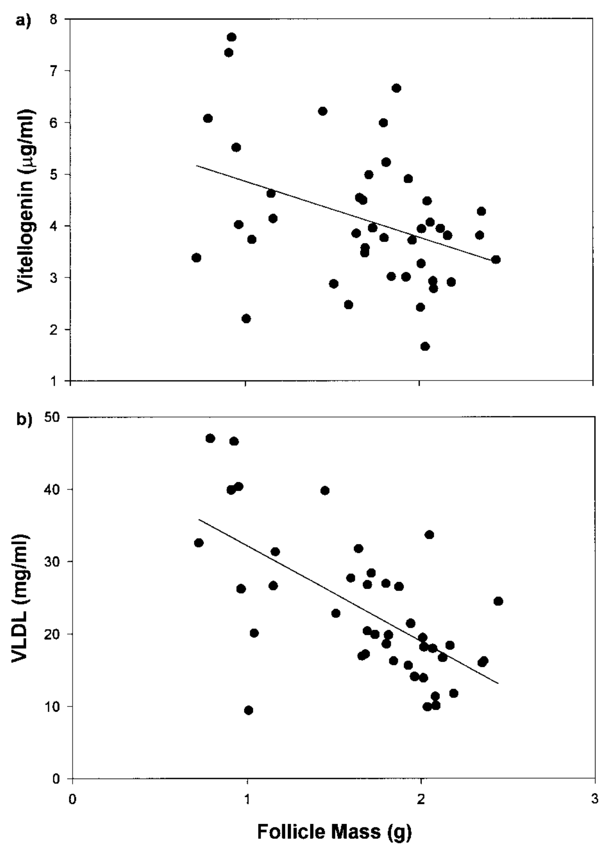

Figure 3. Relationship between total yolky follicle mass and (*a*) plasma vitellogenin and (*b*) plasma very low density lipoprotein (VLDL) concentrations for female starlings with either a full follicle hierarchy or subsequent to this stage (i.e., during the period with no new follicle recruitment).

Table 1: Frequency of occurrence (%) of large yolky follicles (F_1–F_4) in the ovaries of female starlings during the laying cycle

	One Egg	Two Eggs	Three Eggs	Four Eggs	Five Eggs
F_1	100	100	90	17	0
F_2	100	91	20	0	0
F_3	100	55	0	0	0
F_4	95[a]	45[a]	0	0	0
n	20	11	10	6	5

[a] Significant decrease ($P<0.01$) in frequency of occurrence of the next developmentally related follicle (e.g., one-egg F_4 vs. two-egg F_3; see text for details).

Diurnal Variation and Short-Term Repeatability of Plasma Yolk Precursors

Timing of blood sampling had a significant effect on plasma VTG ($F_{1,10}=4.78$, $P<0.05$) and VLDL ($F_{1,10}=8.17$, $P<0.025$) concentrations in the morning sample but not in the evening sample ($P>0.10$; controlling for individual variation by including the plasma concentration of the precursor at the other sampling time as a covariate in multiple regression). Plasma VTG concentrations increased on average by 1.80 μg/mL (48% of the mean value) and plasma VLDL concentrations by 3.9 mg/mL (39%) between 10:00 A.M. and 12:00 P.M. However, for VTG, there was no difference in mean values comparing morning with evening samples ($F_{1,13}=2.40$, $P>0.10$) or in relation to handling or confinement of birds ($P>0.20$; Fig. 4). In contrast, plasma VLDL did show significant diurnal variation, with morning values being on average 14.2 ± 1.1 mg/mL lower than evening values ($F_{1,13}=165.6$, $P<0.001$; Fig. 4). There was also a significant handling effect on VLDL, with nonmanipulated birds sampled in the morning having higher plasma VLDL concentrations than birds held overnight but sampled in the morning ($F_{1,30}=8.58$, $P<0.001$). However, the handling effect was not entirely responsible for the diurnal variation in plasma VLDL, as VLDL concentrations of nonmanipulated birds in the morning were significantly lower than those of birds independently sampled on the evening of their first egg ($F_{1,34}=6.53$, $P<0.025$; Fig. 4).

Within individuals, there was significant repeatability of both precursors comparing morning and evening values (VTG, $F_{1,10}=9.52$, $P<0.025$; VLDL, $F_{1,11}=28.02$, $P<0.001$, controlling for the effect of morning bleed time). Forty-nine percent and 72% of the total variation in VTG and VLDL, respectively, was caused by differences between individuals.

Long-Term Repeatability of Yolk Precursors (between Clutches)

A total of 29 females were blood sampled during both their first and replacement clutches, although most of these individuals ($n=16$) were sampled at different egg stages in the two clutches (e.g., after laying their third and fifth egg, respectively). Mean interval between blood samples was 13.3 ± 1.3 d. Controlling for stage of laying, using residual analysis, plasma VTG was significantly repeatable between clutches ($F_{1,27}=8.67$, $P<0.01$; Fig. 5), with individual accounting for 50.4% of the total variation in VTG (nested ANOVA, $F_{28,57}=3.03$, $P<0.01$). In contrast, plasma VLDL was not repeatable between clutches ($P>0.30$; Fig. 5).

Restricting this analysis to birds that were sampled at the same stage of egg laying in the two successive clutches ($n=13$) gave similar results. Mean interval between blood samples for these females was 14.5 ± 1.5 d. VTG was significantly repeatable between clutches ($F_{1,9}=13.08$, $P<0.01$; controlling for stage of laying), with individual females explaining 76.3% of the total variation ($P<0.001$), whereas VLDL was not repeatable ($P>0.15$). Finally, we confirmed that this result was not an artefact of birds sampled late in the clutch (five-egg

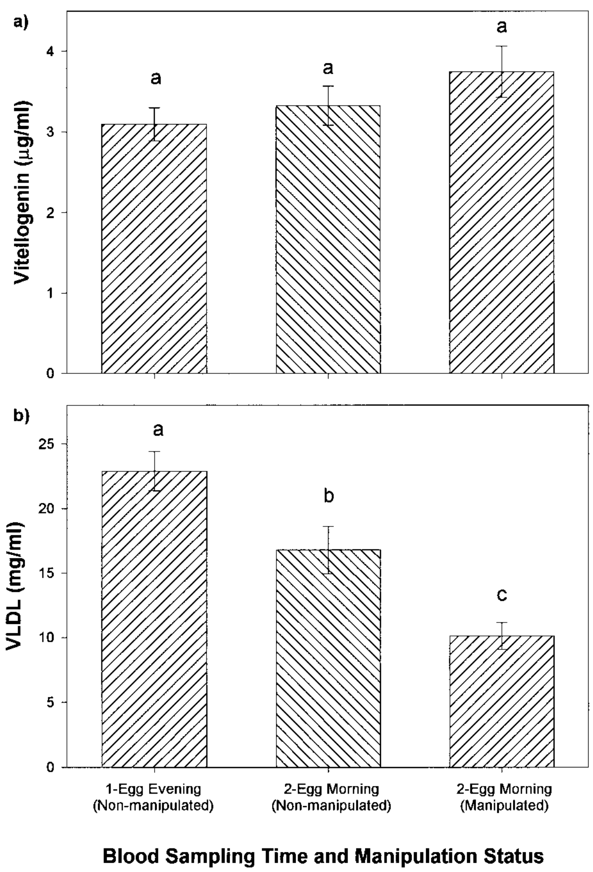

Figure 4. Variation in (*a*) plasma vitellogenin and (*b*) plasma very low density lipoprotein (VLDL) concentrations with time of day and handling stress caused by manipulation (holding females in nestbox overnight). Columns with different letters are significantly different ($P<0.05$). Values are means ± 1 SE.

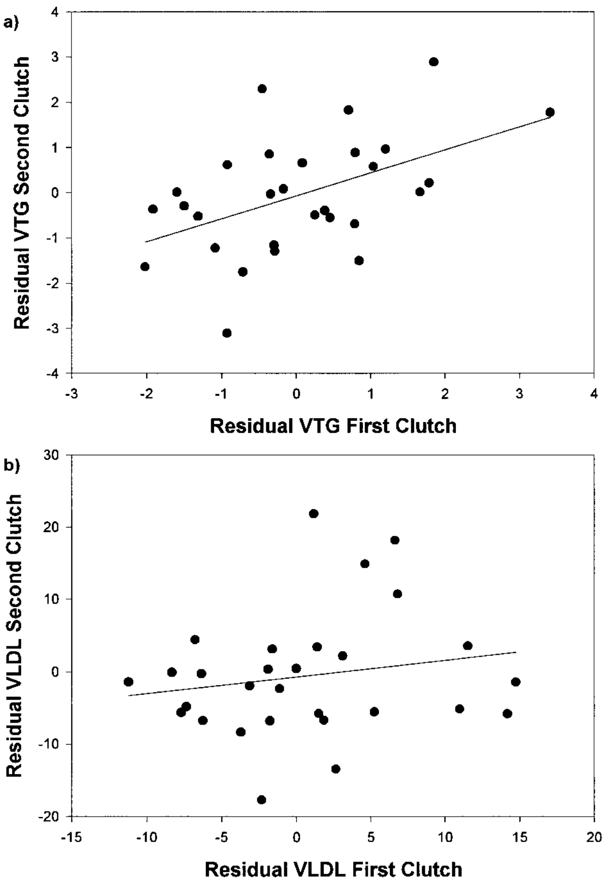

Figure 5. Repeatability of (*a*) plasma vitellogenin (VTG) and (*b*) plasma very low density lipoprotein (VLDL) concentrations (mg/mL) in female starlings sampled during their first and replacement clutches. Values are residuals controlling for variation in stage of oviposition.

stage) always having low VTG values by restricting the analysis to birds sampled at the two- and three-egg stage. First-clutch VTG concentrations were still a significant predictor of second-clutch VTG concentrations ($F_{1,7} = 6.49$, $P < 0.05$), with individuals accounting for 72.5% of the total variation ($F_{8,10} = 6.27$, $P < 0.001$).

Discussion

As expected, there was very large variation in total follicle mass over the 9-d period of yolk formation in the European starling. Although the structure of the ovarian follicular hierarchy has been studied empirically in only a few nondomesticated birds (Etches and Petitte 1990), the pattern of variation in starlings was identical to that previously described for models of egg development based on egg composition data (e.g., Ojanen 1983; Williams and Ternan 1999). At the broad scale, plasma yolk precursor concentrations (i.e., the pool of precursors available to the developing follicles) matched this variation in total follicle mass (see Fig. 1). However, there was evidence for mismatching between supply and demand at two levels. (1) Once the full follicle hierarchy (F_1–F_4) was established, yolk precursor concentrations continued to increase even though follicle mass decreased (with no new follicles being recruited), and yolk precursors concentrations only declined once follicle development was completed. (2) In birds with a full follicle hierarchy, there was a negative relationship between plasma yolk precursor concentrations and total follicle mass.

Yolk Precursor Supply and Follicular Demand

Yolk precursor concentrations were only elevated during the phase of RYD, so European starlings do not start to produce yolk precursors in advance of RYD in readiness for the onset of egg formation, as occurs with some other components of the reproductive system and with male gonads (Williams 1999) to allow for small-scale, annual, or habitat-related adjustments of laying date caused by locally operating supplementary cues for timing of breeding (Wingfield and Farner 1993). This suggests that onset of yolk precursor production can occur sufficiently rapidly (see below) to not constrain onset of laying once local conditions are favourable. The highly specific relationship between timing of yolk precursor production and RYD in starlings contrasts with data from the domestic hen (Redshaw and Follett 1976), where plasma vitellogenin started to increase 4–6 wk before the first oviposition, even though RYD only takes, on average, 8 d per follicle in the hen (Etches 1996).

In the starling, onset of yolk precursor production was tightly coupled to the onset of RYD, with a rapid increase in plasma concentrations almost immediately after appearance of the first yolky follicle to values close to those observed in females with a full follicular hierarchy. Precursor concentrations then did not decline until all follicles had completed their growth. High plasma–yolk precursor concentrations, typical of birds with a full follicle hierarchy, were maintained even though plasma estradiol concentrations (i.e., the signal driving precursor production) probably decrease markedly from the one-egg stage to clutch completion (Sockman and Schwabl 1999) because of changes in steroidogenesis as individual follicles mature from the F_4 to F_1 stage (Bahr et al. 1983). In rainbow trout (*Salmo gairdneri*), uptake rates of yolk precursor into ovarian follicles cultured in vitro is positively related to the concentration of vitellogenin in the surrounding medium ovarian (Tyler et al. 1990). Furthermore, Tyler et al. (1990) showed that maximum uptake rates in cultured oocytes occurred at vitellogenin concentrations equivalent to physiological levels recorded during the latter stages of vitellogenic growth. In zebra finches (*Taeniopygia guttata*), experimental reduction of circulating vitellogenin concentrations results in decreased yolk and egg size (Williams 2000). These observations suggest that high concentrations of VTG and VLDL are necessary to maintain rapid growth of ovarian follicles (i.e., to sustain receptor activities at

V_{max}). The benefits of maintaining high precursor concentrations (e.g., high follicular growth rates) presumably outweigh the costs of mismatching between precursor supply and demand (i.e., maintaining elevated precursor concentrations while follicular demand is decreasing).

This hypothesis does not account for the rise in the concentrations of both yolk precursors between the attainment of a full follicular hierarchy and the ovulation of all but the last yolky follicle. However, plasma concentrations would be expected to rise if the liver maintained a constant supply of precursors while the number of follicles taking up precursor declined. This might also explain the rise in the plasma concentrations of the yolk precursors throughout the morning of the day the second egg was laid: ovulation of the F_1 follicle would lead to a transient reduction in precursor demand and, therefore, a rise in plasma concentrations. Only half of the females recruited a new follicle on the morning the second egg was laid (the frequency of F_4 follicles at the two-egg stage), and so this ovulation would generally mark a decrease in the number of growing follicles. However, constant production of yolk precursors by the liver in the face of declining ovarian demand does suggest mismatching of supply and demand. Perhaps, as yolk precursor production is a steroid-driven process and steroids are relatively slow acting, it may not be possible to make fine-scale temporal adjustments (see also Redshaw and Follett 1976). The negative relationship between plasma precursor concentrations and the mass of the yolky follicles that we found for female starlings with a full follicular hierarchy is analogous to the findings from another study of this species. Christians and Williams (2001) reported a negative correlation between plasma vitellogenin concentrations and mass of yolk protein and lipid (measured in first-laid eggs), suggesting that high uptake rates may yield large follicles (and hence yolks) but may also deplete the circulating pool of vitellogenin.

Follicular Atresia and Clutch Size Determination

Many studies that model investment in egg production assume that the number of follicles that undergo rapid yolk development is equal to the number of eggs laid (e.g., Ojanen 1983; Houston et al. 1995; Williams and Cooch 1999). However, it is known that because of follicular atresia, the number of eggs laid may be less than the number of oocytes recruited into RYD (e.g., Hamman et al. 1986; Krementz and Ankney 1986; Haywood 1993), and this will increase the actual costs of egg production. Although we did find evidence of atresia in starlings, it only occurred during the early stages of follicular development at the first and second egg stage, and only small follicles (F_4 and F_3) became atretic. Thus, recruitment of additional follicles that are not ovulated is unlikely to substantially increase costs of egg production. We found no evidence for burst atresia, that is, atresia of a full follicle hierarchy remaining at cessation of oviposition, as has been reported in chickens (Johnson 1999).

The timing of follicular atresia we observed (i.e., between the one-egg and three-egg stages) is consistent with experiments that have found that European starlings will only lay extra eggs in response to egg removal if eggs are removed on the morning the second egg is laid or earlier (Meijer 1993; Christians 2000). In other words, clutch size must be determined sometime between the laying of the second and third eggs. Our data also support the suggestion of Haywood (1993) that atresia plays a role in determination of clutch size: clutch size is determined by the number of follicles recruited into the hierarchy that complete RYD and are ovulated rather than directly by differences in the number of follicles recruited. However, Haywood (1993) argued that in zebra finches, variation in atresia of follicles in their third or fourth day of development (i.e., F_1 and F_2 follicles) explained clutch size variation, whereas we did not observe atresia of these larger follicles.

Diurnal Variation in Yolk Precursor Concentrations

Plasma vitellogenin concentrations were not significantly different in females sampled at two different times of day: approximately 0–2 and 12–15 h after ovulation. Redshaw and Follett (1976) also found no evidence for a daily rhythm of circulating vitellogenin in the domestic hen, and this supports the idea that birds are maintaining maximal vitellogenin concentrations both throughout the day as well as day to day during RYD. In contrast, plasma VLDL showed marked variation with time of day: circulating concentrations were on average 27% lower in nonmanipulated birds sampled in the morning compared with birds sampled in the evening. A limitation of our method of using total triglycerides as an index of VLDL is that this also measures non-yolk-targeted VLDL (see Vanderkist et al. 2000). However, in nonbreeding birds from a range of species, total triglycerides only vary between 0.5 and 3.0 mg/mL in relation to feeding, short-term (overnight) fasting, and even long-term fasting (e.g., Jenni-Eiermann and Jenni 1996; Williams et al. 1999; Vanderkist et al. 2000). Furthermore, mean plasma triglyceride concentrations in nonlaying starlings captured in the evening at the beginning of the overnight fast are only 3.8 ± 1.6 mg/mL (Christians and Williams 1999*b*) Thus, the difference in plasma VLDL in starlings between morning and evening (on average 6.1 mg/mL) cannot be accounted for by changes in non-yolk-targeted VLDL but must reflect changes in plasma concentrations of yolk-targeted VLDL (VLDLy). Two possible explanations are either that females are metabolizing VLDLy during their overnight fast or that females are not able to maintain VLDLy production overnight when not feeding. VLDLy is known to be poorly metabolized by lipoprotein lipase in vitro in the laying hen (Griffin et al. 1982; Schneider et al. 1990). This is thought to limit its use as an energy source for general metabolism, or uptake into adipose tissue, and would not support the first hypothesis. Nevertheless, it does appear that there might be a trade-off between supply of VLDL for yolk formation and that required for the female's own metabolic needs. This is supported by the fact that diurnal variation in plasma VLDL was more pronounced in females exposed to handling stress overnight, which again would have increased the female's own metabolic demands.

Repeatability of Yolk Precursor Concentrations

This study, as well as several recent studies (Williams and Christians 1997; Christians and Williams 2001; Vanderkist et al. 2000), has shown that circulating concentrations of yolk precursors vary markedly among individuals (four- to eightfold) and that this variation is only weakly related to yolk or egg mass ($r^2 = 11\%$–30%). This could be explained by the fact that yolk precursor production is not costly, as some individuals maintain much higher concentrations than other individuals with little apparent benefit. This, in turn, suggests that circulating yolk precursor concentration is a highly variable and neutral trait. In contradiction to this view, we found a high level of repeatability for both precursors in the short term (24 h) and for vitellogenin in the long term, over about 14 d in successive breeding attempts. Repeatability of measures of performance during egg production (e.g., egg mass; Williams 1996) as well as repeatability of precursor concentrations suggest that there are large-egg and small-egg phenotypes, as well as high-yolk-precursor and low-yolk-precursor phenotypes. Why there is so little concordance between these phenotypes, given the apparently clear-cut mechanistic relationship between yolk precursors and egg production, remains enigmatic.

Conclusion

Our original hypothesis was that there would be some quantitative matching between yolk precursor supply (the plasma pool) and demand for follicular growth (measured as the mass of yolky follicles). In particular, we predicted that plasma concentrations of the yolk precursors (an index of the pool of precursors available to the developing follicles) would be highest on the day(s) of greatest follicular mass and also that it would be highest in individuals with the largest follicle mass, independent of stage of egg production. At the broad scale, there was evidence for economy of design in that yolk precursor concentrations were only elevated specifically during the period of rapid yolk development. However, at the population level, there was mismatching in that yolk precursor concentrations were highest on the last day of follicle development (when only a single F_1 follicle was present) and total follicle mass was less than half that of birds with a complete follicle hierarchy. This might reflect a functional constraint: the plasma precursor pool must remain high to support high uptake rates of the last developing follicle (sensu Tyler et al. 1990). In addition, total follicle mass was actually negatively correlated with yolk precursor concentrations, suggesting that, at the individual level, demand can exceed supply.

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