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ORIGINAL PAPER

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Interindividual variation in yolk mass and the rate of growth of ovarian follicles in the zebra finch (*Taeniopygia guttata*)

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Abstract The amount of resources invested in an individual egg yolk must be determined by its rate of growth and/or the duration of growth. We examined interindividual variation in the growth rate of yolks by injecting radiolabeled amino acid into breeding female zebra finches and measuring the activity associated with protein in the yolks of eggs laid subsequently. We predicted that (1) there would be a positive correlation between yolk mass and the rate of uptake of activity into the yolk; and (2) there would be a negative correlation between clutch size and the amount of activity taken up by each of the follicles due to competition between follicles for circulating yolk precursors. The rate of uptake of activity by the yolks was positively related to yolk mass ($r^2 = 0.24$, 0.35 and 0.50 for the yolks of the third-, fourth- and fifth-laid eggs, respectively), suggesting that interindividual variation in yolk mass is due, at least in part, to variation in the rate of follicle growth. However, we found no evidence of a trade-off between yolk size and number. The uptake of activity was generally repeatable between breeding attempts (repeatability = 0.23–0.44), as was mean yolk mass (repeatability = 0.35), suggesting that these traits are characteristics of individual females.

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Abbreviations *dpm* disintegrations per minute · *RYD* rapid yolk development · *VLDL* very-low-density lipoprotein

Introduction

Natural selection acts on variation within species, and thus studying intraspecific variation in physiology and life history parameters is necessary to understand the evolution of such traits (Bennett 1987; Bradley and Zamer 1999). Little is known about the sources of interindividual variation in avian egg size (Christians and Williams 2001) despite extensive knowledge of the physiological mechanisms involved in egg production in domesticated species (Etches 1996). To determine the mechanistic basis of egg size variation, it is useful to examine the various components of the egg separately since the yolk develops in the ovary whereas the albumen and shell are formed in the oviduct (Etches 1996). In birds, the vast majority of the mass of the yolk is deposited in a relatively short period of time compared to other oviparous vertebrates; in some passerines rapid yolk development (RYD) may last only 3 days per yolk (Ricklefs 1974). During RYD the liver produces two main yolk precursors, vitellogenin and yolk-targeted very-low-density lipoprotein (VLDL), which are secreted into the circulation (Wallace 1985). Vitellogenin and VLDL are taken up from blood by the growing follicles of the ovary via receptor-mediated endocytosis (Griffin and Hermier 1988; Shen et al. 1993), where they provide the primary sources of yolk protein and lipid, respectively. The growth of the ovarian follicles occurs in a hierarchy (Fig. 1), and at most one follicle reaches maturity and ovulates each day.

Clearly, variation in the mass of ovarian follicles at ovulation (and hence in yolk mass) must result from variation in the rate of growth of the follicles and/or the

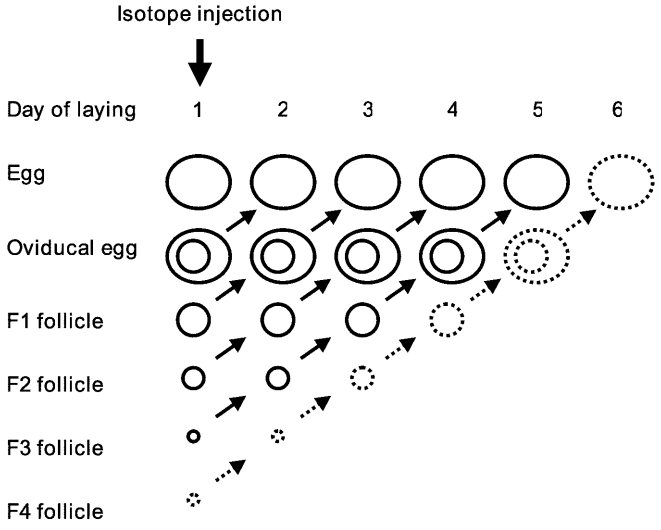

Fig. 1 The timing of isotope injection relative to the timing of yolk growth in a five-egg (*solid symbols*) and six-egg (*dashed symbols*) clutch

duration of their growth. Although the relationship between the duration of RYD and yolk mass has been studied within domesticated species (Bacon and Koontz 1971; Imai 1983), little work has been done on other species (but see Birkhead and Del Nevo 1987; Hatchwell and Pellatt 1990). Furthermore, the importance of interindividual variation in follicle growth rate in determining yolk mass has yet to be examined.

The goal of this study was to investigate whether large yolks are formed more rapidly than small yolks (versus taking longer to form), using protein deposition as a measure of growth rate. We injected radiolabeled amino acid into breeding female zebra finches (*Taeniopygia guttata*), and examined the activity associated with protein in the yolks of eggs laid subsequently. We predicted that, if larger yolks were the result of more rapid rates of protein deposition, there would be a positive relationship between the rate of uptake of activity into the yolk and yolk mass. Measuring the rate of protein deposition also allowed us to examine the potential trade-off between the number of growing yolks and yolk size. Thus, our second prediction was that, if there were a trade-off between yolk number and size due to competition between follicles for circulating yolk precursors, the rate of uptake of activity per yolk would decrease with increasing clutch size. Finally, because reproductive parameters such as egg size and clutch size are generally repeatable within individual females (Boag and Noordwijk 1987), we predicted that the rate of protein deposition would be repeatable between breeding attempts.

Materials and methods

General

A captive-breeding population of zebra finches was maintained in controlled environmental conditions (temperature 24–28°C; humidity 35–55%; constant light schedule, 14L:10D, lights on at 0700 h), with birds kept in single-sex cages prior to experiments. All birds were provided with mixed seed (white and panicum millet; 11.7% protein, 0.6% lipid, and 84.3% carbohydrate), water, grit, and cuttlefish bone ad libitum and received a multi-vitamin supplement in the drinking water once per week. During breeding, birds also received a daily egg-food supplement (20.3% protein, 6.6% lipid) and were housed in pairs in cages (51 cm×39 cm×43 cm), each with an external nest box (14 cm×14 cm×20 cm). Females were introduced to the breeding cages in the morning and a single male partner was added within 3 h. Nest boxes were checked daily for eggs. Eggs are generally laid at daily intervals, but females occasionally "skip" a day, i.e. lay two eggs ca. 48 h apart (Williams 1996). Clutch size was therefore defined as the number of eggs laid, allowing interruptions of 1 day.

Injection of radiolabeled amino acid

Thirty-one females were paired with males. On the day a female laid her first egg (i.e. one-egg stage), she was weighed (± 0.1 g), a blood sample was taken for measurement of plasma vitellogenin levels (see below), and the female was injected intraperitoneally with 1 μCi L-[U-^{14}C] serine (151 mCi mmol^{-1}; Amersham Pharmacia Biotech) in 100 μl phosphate-buffered saline. Figure 1 shows the timing of isotope injection relative to the stage of growth of the various ovarian follicles. In chickens, radiolabeled amino acids are incorporated rapidly into newly synthesized vitellogenin (~15 min; Jost et al. 1978) and the apoprotein components of VLDL (< 2.5 h; Evans and Burley 1987). We used radiolabeled serine because vitellogenin, the primary source of yolk protein, has an unusually high serine content and therefore serine would selectively (but not exclusively) label this protein (Wang and Williams 1982). Since ^{14}C-serine would also label all other newly synthesized protein, we refer to "protein uptake" by the ovary. All ^{14}C injections were performed between 5 h and 7.5 h after lights-on to ensure that isotope was administered after that day's ovulation (i.e. the ovulation of the yolk of the second egg); 91% of eggs are laid within 1–3 h of lights-on (Williams 1996) and ovulation occurs 30–45 min after oviposition of the preceding egg (Etches 1996).

We repeated the experiment a second time using the same females as in the first experiment so that the repeatability of reproductive and physiological parameters could be assessed. The interval between isotope injection in the first and second experiments was approximately 9 weeks. In the second experiment the time of oviposition (± 0.5 h) was recorded as an estimate of the time of ovulation (Hammond et al. 1980; Calvo and Bahr 1983; Etches 1996).

Measurement of activity in the yolks of eggs laid after isotope injection

The first five eggs laid by each female were removed on the day they were laid, weighed (± 0.001 g), and replaced with "dummy" eggs (i.e. eggs from other, non-manipulated females). Within 48 h of laying, yolks were separated from eggs, weighed fresh, and placed in a 1.5-ml micro-tube. To measure only protein-associated activity (i.e. incorporated into ovarian follicles via receptor-mediated processes), rather than activity associated with free serine, we precipitated protein from the yolks by mixing each yolk with 500 μl of 7% trichloroacetic acid solution, leaving the suspension on ice for 10 min, and centrifuging at 13000 rpm for 10 min. The resulting supernatant was discarded and the precipitate was rinsed three times with water, resuspended in 1 ml water and centrifuged again at 13,000 rpm for 10 min. The resulting precipitate was rinsed once and then frozen.

Yolk protein was later thawed and resuspended in 15 ml scintillation cocktail (Amersham biodegradable counting scintillant). Samples were left for 3 days (first experiment) or 6 days (second experiment) to improve the suspension of yolk protein in the cocktail, and were placed in the dark at least 24 h prior to counting to reduce interference due to chemiluminescence. All samples were counted on a Beckman LS6500 scintillation counter for 5 min.

Counts per minute were converted to disintegrations per minute (dpm) using a quench curve constructed with zebra finch yolks.

Measurement of plasma vitellogenin concentrations

The amount of activity incorporated into ovarian follicles was not simply a function of the rate of protein uptake. There were large amounts of unlabeled yolk precursors in the circulation at the time of isotope injection, and these would have reduced the specific activity of the newly labeled protein and potentially competed with labeled precursors for the receptors on the oocyte membrane responsible for protein uptake. The primary source of yolk protein is vitellogenin (Wallace 1985), and thus we measured the circulating concentrations of this precursor at the time of ^{14}C injection to account for variation in unlabeled precursor pools.

Plasma levels of vitellogenin were measured using the vitellogenic zinc method developed for the domestic hen *Gallus gallus domesticus* (Mitchell and Carlisle 1991) and validated for passerines (Williams and Martyniuk 2000). This method measures the concentration of zinc bound to vitellogenin (i.e. vitellogenic zinc) as an index of the concentration this protein (Mitchell and Carlisle 1991). The inter-assay coefficient of variation for the assays performed in this study was 7.6% ($n = 8$).

Statistical analyses

To reduce variation in the number and stage of ovarian follicles undergoing RYD at the time of isotope injection, we restricted analyses to females that laid five or more eggs and that did not skip a day while laying the first five eggs. The number of females that laid fewer than five eggs or that skipped within the first five eggs was too small to allow meaningful comparison with the other females. In the first experiment, 27 females laid five or more eggs without skipping, whereas the sample size in the second experiment was 26. In the second experiment, the time of ovulation of the fifth yolk was not obtained for one female, and the plasma concentration of vitellogenin was not obtained for another, and so sample sizes are reduced slightly for analyses involving these parameters. The results of the first and second experiments were very similar and so only the results of the second experiment are presented (except for the repeatability results which incorporate data from both experiments). We present the results of the second experiment since the timing of ovulation was measured in this experiment but not in the first.

Means ± standard errors are presented, except where stated. To estimate the association between two variables, we calculated Pearson correlation coefficients (CORR procedure; SAS Institute 1990). To statistically control for variation in additional variables, we used the PARTIAL statement of the CORR procedure (SAS Institute 1990); in such cases partial correlation coefficients are presented. Subscripts of the correlation coefficients denote sample sizes. An index of body condition was calculated using the residuals of the regression of female mass at pairing on body size (Williams 1996); body size was calculated using the first principal component from a principal components analysis combining the lengths of the bill and tarsus (PRINCOMP procedure; SAS Institute 1989).

Twenty-three females laid five or more eggs without skipping in both the first and second experiments and were used for repeatability analyses. Between-experiment repeatability was calculated following Lessells and Boag (1987). We use R to denote repeatability to avoid confusion with correlation coefficients (r).

Results

Variation in mean egg mass, mean yolk mass and timing of oviposition

We examined variation between females and therefore use the terms "mean egg mass" and "mean yolk mass" to refer to the mean within clutches (i.e. within females). Mean yolk mass showed large variation between females, the smallest mean yolk mass (218 mg) being only 71% of the largest (308 mg). The mean value for all females was 259 ± 4 mg ($n = 26$). Mean egg mass showed similar variation, ranging from 0.96 g to 1.49 g (mean = 1.13 ± 0.02 g). Mean yolk mass was not correlated with mean egg mass ($r_{26} = 0.29$, > 0.1), although the relationship may have been obscured by one outlier with an extremely large egg mass and average yolk mass; when this female was excluded from the analysis, mean yolk mass was related to mean egg mass ($r_{25} = 0.45$, $P = 0.024$).

The between-female variation in mean yolk mass was not related to female mass at the one-egg stage ($r_{26} = 0.06$, $P > 0.2$). Similarly, mean egg mass was not related to female mass at the one-egg stage ($r_{26} = 0.34$; $P = 0.09$), although removing the outlier described above strengthened the relationship ($r_{25} = 0.48$, $P = 0.014$). Neither mean yolk mass nor mean egg mass was explained by variation in body size, condition, the plasma concentration of vitellogenin at the one-egg stage, or the number of days between pairing and the day the first egg was laid ($|r| < 0.11$, $P > 0.2$ in all cases). Ten, 14 and 2 females laid clutches of five, six or seven eggs, respectively, and the clutch-size classes did not differ in mean yolk mass ($F_{2,23} = 0.60$, $P > 0.2$) or mean egg mass ($F_{2,23} = 0.22$, $P > 0.2$).

Eighty-seven percent (90/103) of eggs were laid within 1 h of lights-on and 95% (98/103) were laid within 2 h. The mean daily interval was calculated for each female as the average of the intervals between eggs 2 and 3, between eggs 3 and 4, and between eggs 4 and 5. The average of this value among all females was 24.00 ± 0.06 h (range 22.97–24.76). Mean daily interval was not related to mean egg mass ($r_{25} = 0.13$, $P > 0.2$) or mean yolk mass ($r_{25} = 0.21$, $P > 0.2$).

Activity incorporated into yolks

The amount of activity in the yolks of first- and second-laid eggs was approximately two orders of magnitude lower than that observed in the third-, fourth- and fifth-laid eggs (Table 1). This effect of laying sequence was highly significant ($F_{4,99} = 483.32$, $P < 0.0001$), as was the variation between females in the amount of activity

Table 1 Variation in the amount of activity incorporated into yolks with laying sequence. Values with the same superscript are not significantly different (*dpm* disintegrations per minute)

Egg	Amount of activity in yolk (dpm×10^3)
1	0.068 ± 0.001^a
2	0.070 ± 0.003^a
3	14.2 ± 0.6^b
4	13.6 ± 0.5^b
5	8.5 ± 0.4^c

incorporated into the yolks ($F_{25,99}=3.53$, $P<0.0001$, including both sequence and female as terms in a general linear model; GLM procedure, SAS Institute 1989). The extremely low activity in the yolks of first eggs indicates that contamination from radioisotope administered in the first experiment was trivial. Similarly, the low activity in the yolks of second eggs demonstrates that cross-contamination between albumen and yolk was negligible since radioisotope was injected while the albumen of the second egg was being deposited (see Fig. 1).

The rate of uptake of activity into each yolk was calculated by dividing the amount of activity in the yolk by the number of hours between isotope injection and the time of ovulation. Uptake rates of the yolks of the third, fourth and fifth eggs were highly correlated with each other (yolks 3 and 4: $r_{26}=0.77$, $P<0.0001$; yolks 4 and 5: $r_{25}=0.84$, $P<0.0001$; yolks 3 and 5: $r_{25}=0.57$, $P<0.01$). The rate of isotope uptake into the yolk of the third egg was negatively correlated with the mass of the female at the time of isotope injection ($r_{26}=$-0.42, $P=0.034$); this was likely the result of dilution of the labeled serine by the pool of unlabeled amino acid within the female. Female mass at the one-egg stage was not correlated with the rate of isotope uptake in the fourth and fifth yolks, although the trends were negative in both cases ($r_{26}=$–0.30, $P>0.1$, and $r_{25}=$–0.07, $P>0.2$, respectively).

The uptake rate of the yolk of the third egg was positively correlated with the mass of the third yolk (partial $r_{25}=0.49$, $P=0.018$; Fig. 2A). Similarly, the uptake rates of the fourth and fifth yolk were positively correlated with the masses of the fourth (partial $r_{25}=0.59$, $P<0.01$; Fig. 2B) and fifth yolks (partial $r_{24}=0.71$, $P<0.001$; Fig. 2C), respectively. These analyses control for the mass of the female and the plasma concentration of vitellogenin at the one-egg stage to account for variation in isotope dilution by endogenous, unlabeled serine, and variation in the specific activity of labeled vitellogenin, respectively. The relationship between mean yolk mass and the rate of uptake of activity into the third yolk was not significant (partial $r_{25}=0.30$, $P>0.1$), controlling for female mass and plasma vitellogenin concentration. However, the correlation between uptake rate and mean yolk mass was stronger and significant for the fourth (partial $r_{25}=0.48$, $P=0.022$) and fifth yolks (partial $r_{24}=0.55$, $P<0.01$). The uptake rates of the third, fourth and fifth yolks were not related to mean egg mass, controlling for female mass and plasma vitellogenin concentration (partial $|r|<0.2$; $P>0.2$ in all cases).

The effect of clutch size on the rate of uptake of activity into the third yolk was not significant ($F_{2,19}=2.62$, $P=0.1$), and there was no evidence of a linear decrease with increasing clutch size; clutches of six had a lower mean rate than did clutches of five or seven (Table 2). Similarly, uptake rates in fourth and fifth yolks did not differ between clutch size classes (fourth yolk: $F_{2,19}=0.28$, $P>0.2$; fifth yolk: $F_{2,18}=1.06$, $P>0.2$; Table 2). In these analyses, the mass of the yolk, the mass of the female and the plasma concentration of vitellogenin at the one-egg stage were included as covariates in a general linear model (SAS Institute 1989).

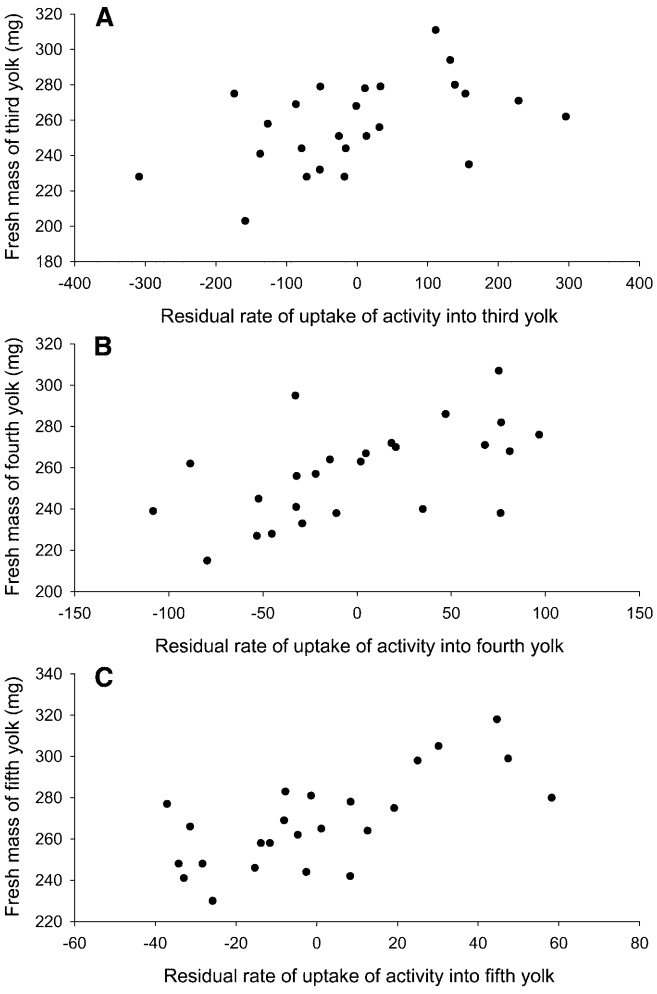

Fig. 2 Relationships between fresh yolk mass and the rate of uptake of activity for the yolks of the third (**A**), fourth (**B**) and fifth (**C**) eggs. Uptake rates are shown as the residuals of a multiple regression of uptake rate on body mass and the plasma levels of vitellogenin at the one-egg stage

Table 2 Variation in the rate of uptake of activity into the yolks of the third, fourth, and fifth-laid eggs with clutch size. Uptake rates did not differ significantly between clutch size classes

Clutch size	Rate of uptake of activity (dpm h^{-1})		
	Third yolk	Fourth yolk	Fifth yolk
5	843 ± 42	331 ± 18	125 ± 8
6	731 ± 32	320 ± 14	130 ± 6
7	852 ± 86	347 ± 37	149 ± 15

Repeatability of reproductive and physiological parameters

A female's mean yolk mass was repeatable between the first and second experiment ($R=0.35$, $P=0.046$). The

repeatability of mean egg mass was higher ($R = 0.78$, $P < 0.0001$) than that of yolk mass, and clutch size was also repeatable ($R = 0.38$, $P = 0.035$). The repeatability of female mass at the one-egg stage was high ($R = 0.87$, $P < 0.0001$), but that of the plasma concentration of vitellogenin was less so ($R = 0.43$, $P = 0.019$). Because we did not have estimates of the times of ovulation in the first experiment, we could not calculate the repeatability of uptake rates. However, the absolute amount of activity incorporated into the third and fifth yolk was repeatable (third yolk: $R = 0.44$, $P = 0.015$; fifth yolk: $R = 0.35$, $P = 0.043$), although the repeatability of activity in the fourth yolk was not significant ($R = 0.23$, $P > 0.1$).

Discussion

The goal of this study was to investigate the physiological basis of interindividual variation in yolk mass. We injected breeding female zebra finches with ^{14}C-serine and measured the amount of activity in the protein of yolks laid subsequently. Radiolabeled amino acids are rapidly incorporated into vitellogenin, the primary source of yolk protein (Wallace 1985), when injected into vitellogenic birds (Jost et al. 1978), and labeled vitellogenin is taken up selectively into growing follicles in chickens (Cutting and Roth 1973), rainbow trout *Salmo gairdneri* (Tyler et al. 1988, 1990b) and the South African clawed toad *Xenopus laevis* (Wallace and Dumont 1968). Thus, the activity we measured was an index of the amount of protein taken up by the ovarian follicles specifically through receptor-mediated endocytosis. To our knowledge, this is the first study to relate interindividual variation in the rate of follicle growth to variation in yolk size in any oviparous vertebrate.

The rate of uptake of activity by the yolks was positively related to yolk mass, suggesting that interindividual variation in yolk mass is due, at least in part, to variation in the rate of follicle growth. The correlation between uptake rate and mean yolk mass was stronger for the fourth and fifth yolks (which had over one and two days of growth to complete after isotope injection, respectively; see Fig. 1), than it was for the third yolk (which had less than one day between isotope injection and ovulation). Thus, variation in uptake rate in the early and middle stages of RYD may be more important in determining yolk mass than uptake rate in the final stage. This does not mean that uptake rates decrease in the final stages of RYD; our data do not address this issue. Our data bear on the patterns of interindividual variation in uptake rates, and not on the magnitude of the average uptake rates.

We found no evidence that the number of growing follicles affected yolk mass due to competition between follicles for circulating yolk precursors. Mean yolk mass did not differ between clutches of five, six or seven eggs, and clutch size did not affect the rate of uptake of activity into yolks. However, because of follicular atresia the number of eggs laid may not accurately reflect the number of follicles that initiated RYD (Haywood 1993), or the number of follicles that grew simultaneously. This may explain why we found no evidence of a trade-off between yolk size and number whereas Nager et al. (2000) did demonstrate a trade-off between egg number and quality in the lesser black-backed gull (*Larus fuscus*).

The rate of uptake of activity into the fourth and fifth yolk was correlated not only with the mass of those yolks, but also with mean yolk mass, suggesting that all yolks within a clutch have a similar rate of growth; the correlations among the uptake rates of different yolks are consistent with this proposition. Furthermore, the repeatability of mean yolk mass and the amount of activity incorporated into the third and fifth yolks within females in different breeding attempts indicate that yolk mass and uptake rate are characteristics of individual females. Egg size has been found to be highly repeatable and heritable in a variety of species (Boag and Noordwijk 1987), and so it is possible that the repeatability of uptake rate is also a widespread phenomenon in birds.

If interindividual variation in yolk mass is due to variation in the rate of follicular growth, what limits the rate of uptake of the yolk precursors? The circulating concentrations of vitellogenin might be expected to determine uptake rate (Tyler et al. 1990a). However, in our study there was no relationship between the circulating concentration of vitellogenin and yolk mass; Christians and Williams (2001) actually found this correlation to be negative in free-living European starlings (*Sturnus vulgaris*). A negative relationship is consistent with the hypothesis that yolk mass is determined by the rate of precursor uptake; higher uptake rates lead to larger yolks, but either deplete the circulating pool of vitellogenin or do not allow it to build up. Breeding birds may maintain precursor concentrations above the threshold required to saturate the uptake process (see Tyler et al. 1990a); when exogenous estradiol was administered to breeding European starlings, the plasma levels of vitellogenin were increased but there was no increase in egg or yolk mass (Christians and Williams 1999). However, decreases in precursor concentration below this threshold may lead to a reduction in uptake rates, and hence decreased yolk and egg mass. Using the anti-estrogen tamoxifen, Williams (2000) experimentally reduced vitellogenin levels in breeding zebra finches and found the expected decrease in yolk and egg mass.

Precursor uptake may be limited instead by the number of vitellogenin/VLDL receptors and by their rate of recycling back to the oocyte surface following endocytosis (Griffin and Hermier 1988). Vitellogenin receptors are synthesized prior to RYD in both chickens (Shen et al. 1993) and trout (Davail et al. 1998; Perazzolo et al. 1999), and relocate to the surface of the oocyte at the onset of vitellogenesis (Shen et al. 1993; Bujo et al. 1994). Thus, growing follicles rely on a previously synthesized pool of receptors for their growth (Shen et al. 1993; Davail et al. 1998; Perazzolo et al. 1999) and therefore maximal uptake rates may be determined prior to egg production, and potentially prior

to sexual maturity. In addition to variation in receptor number, variation in receptor quality (e.g. due to mutation, Bujo et al. 1995), could also affect uptake rates. However, the rate of yolk precursor uptake is clearly not fixed within individuals and could also be subject to hormonal adjustment (e.g. Tyler et al. 1991).

The results of this study do not preclude the possibility that some of the variation in yolk mass is due to variation in the duration of RYD. Imai (1983) found that the number of days of rapid growth was positively correlated with follicular volume in chickens, although no relationship between yolk mass and the duration of RYD was found in quail *Coturnix coturnix japonica* (Bacon and Koontz 1971) or free-living common guillemots *Uria aalge* (Hatchwell and Pellatt 1990). In wild species, the duration of RYD may show little intraspecific variation, as found in Adélie penguins *Pygoscelis adeliae*, and Cassin's auklets *Ptychoramphus aleuticus* (Astheimer and Grau 1990). Variation in the time between successive ovulations could also contribute to variation in yolk mass. Egg mass is related to the interval between successive ovipositions in chickens (Etches 1996) and European starlings (Meijer 1992), but this could be due to variation in albumen and/or shell mass rather than yolk mass. In this study, the mean interval between successive ovulations did not explain variation in yolk mass or egg mass, despite variation in interval as great as that observed by Meijer (1992).

In summary, we have presented evidence that interindividual variation in yolk mass is determined, at least in part, by the rate of follicular growth. Furthermore, both the mass and growth rate of yolks are repeatable within females in different breeding attempts, demonstrating that the ovarian uptake of yolk precursors deserves further study as a potential source of interindividual variation in reproductive effort.

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