Vitellogenin dynamics during egg-laying: daily variation, repeatability and relationship with egg size

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Daily variation in circulating levels of the avian yolk precursor, vitellogenin (VTG), throughout the laying cycle was investigated in female zebra finches Taeniopygia guttata and compared with predicted ovarian follicle demand (based on a model of follicular development for this species). In general, the pattern of variation in plasma VTG matched the predicted demand from the developing ovarian follicle hierarchy. Plasma VTG was non-detectable in non-breeders, but increased rapidly with onset of yolk development, remaining high (1.43–1.82 μg/ml, zinc) through to the 3-egg stage. Plasma levels then declined at the 5-egg stage (to 0.78 ± 0.32 μg/ml) and were undetectable at clutch completion. This result is consistent with the hypothesis that yolk precursor production is costly and that selection has matched supply and demand. While inter-individual variation in plasma VTG was marked (e.g. 0.47–4.26 μg/ml at the 1-egg stage), it also exhibited high intra-individual repeatability (r = 0.87–0.93). Finally, we examined the relationship between plasma VTG and primary reproductive effort. While individual variation in plasma VTG was independent of clutch size, laying interval and laying rate, there was a complex, diet-dependent relationship between VTG and egg size, with low plasma VTG levels being associated with both very small (<0.90 g) and very large (>1.15 g) egg sizes.
laying cycle in female zebra finches Taeniopygia guttata, Williams and Christians 1997). In both of these studies, plasma VTG was measured at a single point in the laying cycle (the 1-egg stage) but the experimental diets differed between studies, raising the possibility that the form of the relationship between plasma VTG and reproductive output is diet- (i.e. resource) dependent. These studies also provided no information on how yolk precursor levels vary throughout the laying cycle (but see Challenger et al. 2001) or to what extent they might vary within individuals between breeding attempts (i.e. repeatability of yolk precursor production).

In this paper we first describe the pattern of daily variation in circulating levels of VTG throughout the laying cycle in female zebra finches, and compare this pattern to the predicted demand based on a model of ovarian follicle development for this species. Second, we assess repeatability of VTG, that is, intra-individual variation between different breeding attempts, in relation to inter-individual variation. Third, we examine how diet affects the relationship between inter-individual variation in circulating VTG levels and egg size, clutch size and laying rate) and resolve the contradictory patterns reported in previous studies.

Materials and methods

Animals and husbandry

Zebra finches were maintained in controlled environmental conditions (temperature 19–23°C, humidity 35–55%, constant light schedule of 14L:10D, lights on at 07:00). All birds received a mixed seed diet (Panicum and white millet, 1:3; approximately 11.7% protein and 0.6% lipid by dry mass; Jameson’s Pet Food, Vancouver), water, grit, and cuttlefish bone (calcium) ad lib. Birds also received a multivitamin supplement in the drinking water once per week. When not paired for breeding, the birds were housed in same-sex cages, but were not visually or acoustically isolated from the opposite sex. Breeding pairs were housed individually in cages (61 × 46 × 41 cm) equipped with an external nest box (11.5 × 11.5 × 11.5 cm). Males and females were weighed (+ 0.1 g) at the time of pairing. Data on laying interval and egg and clutch size were obtained by checking the nest boxes daily between 09:00 and 11:00. Clutches were considered complete if no new eggs were laid over two days, and the pair was then returned to non-breeding cages. Females that failed to lay eggs within 15 days were classified as non-breeders and were returned to non-breeding cages. All experiments and animal husbandry were carried out under a Simon Fraser University Animal Care Committee permit (no. 55BB) following guidelines of the Canadian Committee on Animal Care.

Daily variation, individual variation, and repeatability of plasma VTG

Experienced female zebra finches (i.e. birds which had bred at least once previously) were randomly chosen from non-breeder cages, and each female was paired twice with at least 28 days between each breeding attempt. During each breeding attempt each female was randomly paired with an experienced male, and all pairs were provided with 6 g egg-food supplement (a mixture of 62–65 g hard-boiled egg, 13 g cornmeal, 13 g bread crumbs; 30.2% protein and 13.0% lipid by dry mass) daily between pairing and clutch completion (see Williams 1996b) in addition to the normal seed diet. All new eggs were collected on the day they were laid, weighed (to 0.001 g), numbered and substituted with replacement zebra finch eggs (to maintain the birds’ actual clutch size); egg composition analysis was performed on all collected eggs for another experiment. During both breeding attempts, each female was randomly assigned to one of five blood-sample groups which determined when blood sampling (~200 µl from the brachial vein) would occur during the laying cycle: (a) two days after pairing (n = 10), (b) four days after pairing (n = 10), (c) first-egg stage (n = 10), (d) third-egg stage (n = 10), and (e) fifth-egg stage (n = 10). Since individual females varied in timing of clutch initiation, blood samples taken at two and four days after pairing ranged in time relative to the first egg, from zero to 10 days prior to the day the first egg was laid. Therefore, the pre-laying period was divided into two physiologically distinct stages: (1) the early, pre-laying stage (EPL), which was considered to be five or more days prior to the laying of the first egg, and (2) the rapid-yolk development stage (RYD), during which time the newly recruited follicles are undergoing yolk precursor uptake. In zebra finches RYD is assumed to begin about four days prior to the day the first egg is laid (Haywood 1993, Williams and Terman 1999). Randomly chosen females from each blood sample group were also sampled for blood at clutch completion (n = 6). During the second breeding attempt, individual females were sampled for blood again, in all cases at the same time relative to either pairing or laying as in their first breeding attempt (in order to determine repeatability of the yolk precursors). All blood samples were collected between 09:00 and 11:30. Blood sampling had no significant effect on the mass or composition of eggs undergoing RYD at the time of sampling (K. G. Salvante unpubl. data). Females in the 1-egg stage blood-sample group were only paired once, and their eggs were not collected in order to raise chicks for another study. Blood samples were taken from randomly chosen non-breeding females from the same-sex non-breeding cages (n = 11) to obtain non-breeding measures of plasma VTG. In order to assess the extent of ‘matching’ between supply and demand for VTG,
circulating levels of VTG at various stages of the laying cycle were compared to a model of ovarian follicle growth and yolk precursor ‘demand’ previously generated for a 6-egg clutch (for details of the model see Williams and Ternan 1999; units of total energy used in Williams and Ternan 1999 were converted to grams of yolk assuming zebra finch yolk is composed of 75% water, 15% lipid, and 10% protein (K. G. Salvante unpubl. data), and the energy equivalents of 9.4 kcal/g lipid and 4.25 kcal/g protein (Schmidt-Nielsen 1990)).

**Variation in plasma VTG in relation to reproductive effort**

Female zebra finches were paired under the same environmental conditions, and using the same methods as described previously, with two exceptions: (1) all of the females in this part of the study were sampled for blood only on the day that the first egg was laid (1-egg stage), and (2) all of the eggs were weighed and numbered, but not collected. Data from two groups of females were compared: (1) experienced females fed the seed diet supplemented with egg-food (n = 25) and (2) experienced females on a seed only diet (n = 25). Egg size, clutch size, and laying interval were measured in the same manner as previously described.

**Analysis of plasma VTG**

Plasma samples were assayed for VTG using the zinc method developed for the domestic hen (Zinc kit – Wako Chemicals, Virginia, USA; Mitchell and Carlisle 1991), and validated for passerines (Williams and Christians 1997). This method measures total plasma zinc, and then separates the zinc bound to serum albumen from that bound to VTG and very-low density lipoprotein (VLDL) by depletion of VTG and VLDL from the plasma sample by precipitation with dextran sulfate. The depleted plasma sample is then assayed for zinc. Vitellogenic zinc (VTG-Zn) is equal to the difference between total and depleted zinc; VLDL accounts for only 2% of total plasma zinc (Mitchell and Carlisle 1991). The concentration of VTG-Zn is proportional to the plasma concentration of plasma VTG (Mitchell and Carlisle 1991). Total zinc and VTG-Zn were highly correlated (r = 0.843, P < 0.0001). Therefore, total zinc was used as an index of VTG-Zn when there was insufficient plasma to carry out the depletion step necessary to measure VTG-Zn. Intra-assay coefficients of variation (%) determined for a laying hen plasma pool was 2.93% and 3.42% (n = 15 sample replicates) for total and depleted zinc respectively, and inter-assay coefficients of variation were 8.6% and 14.5% respectively (n = 15 assays). All assays were run using 96-well microplates, and measured using a Biotek 340i microplate reader.

**Statistical analysis**

All statistical analyses were performed using SAS (SAS Institute 1989). All non-normal variables, as assessed by the Shapiro-Wilk test for normality (Zarr 1996), were normalized for future analysis through log 10 transformation (although some non-transformed values were used for graphical purposes). The relationships between body mass and reproductive traits (precursor levels, egg size, etc.) were examined by regression of the trait values against body mass. Mass-dependent traits were corrected for body mass by taking the residual values from the regression analyses. ANOVA with Bonferroni adjustment of significance level for multiple comparison tests were used to compare the various blood sample groups when assessing the daily variation in circulating levels of VTG throughout the laying cycle (Rice 1989). The overall significance level was retained as 0.05; each of the 21 comparisons was tested at a significance level of (0.05/21) or 0.002. Nested ANOVA was used to determine the repeatability of the yolk precursor following Lessells and Boug (1987). To determine the relationships between plasma levels of VTG and reproductive traits, ANOVA and regression analyses were performed. All values are given as means ± standard error (unless otherwise stated), all tests are two-tailed, and the overall significance level is P < 0.05.

**Results**

**Daily and individual variation in plasma VTG and reproductive effort**

Mean egg size was positively correlated with female body mass at the 1-egg stage (r = 0.38, F$_{1,50}$ = 6.2, P < 0.001). Plasma levels of VTG, clutch size, and laying interval were all independent of body mass and diet (P > 0.05 in all cases). Individual variation was highly marked both for plasma VTG levels and for reproductive traits; plasma VTG ranged from 0.47 to 4.26 µg/ml at the 1-egg stage, mean egg mass varied almost two-fold in females that were sampled for blood at the 1-egg stage, clutch size ranged from 1 to 11 eggs, and laying interval ranged from 1 to 13 days after pairing. Subsequent analyses of clutch size were limited to clutches with 2–7 eggs because this is the normal range encountered in our captive population (e.g. Williams and Ternan 1999), and because of the small sample size of clutches with only 1 egg or over 7 eggs.

Plasma VTG levels varied markedly throughout the laying cycle (F$_{6,80}$ = 23.13, P < 0.001), mirroring changes in follicle demand based on estimated yolk transferred to the developing follicles (Fig. 1). Plasma VTG levels in non-breeding females were not significantly different from zero, and also were not different from plasma levels at clutch completion (P > 0.25 in
Prior to the predicted onset of RYD, plasma VTG levels increased slightly, but not significantly ($P > 0.002$), from the non-breeding stage to the early pre-laying stage. With the onset of RYD there was a sharp increase in plasma VTG to $1.43 \pm 0.51$ µg/ml, and plasma levels remained high through the 1-egg and 3-egg stages (no difference between these three stages, $P > 0.80$ in all cases; Fig. 1). Circulating levels of the yolk precursor then decreased significantly between the 3-egg and 5-egg stage ($P < 0.0015$), but were still significantly higher than zero at the 5-egg stage ($P < 0.00025$). By clutch completion (CC), two days after the last egg was laid, plasma VTG levels were again not significantly different from zero ($P > 0.90$).

**Repeatability of VTG**

A total of 28 females were blood sampled during both their first and second breeding attempts and 12 of these birds were sampled at the same stage of laying in the two clutches. Controlling for laying stage, plasma VTG was highly repeatable within individuals between their first and second breeding attempts ($r = 0.87$, $F_{27,28} = 12.3$, $P < 0.001$, $n = 28$, Fig. 2a) with individual explaining 85.0% of the total variation in plasma VTG. Similar results were obtained when analysis was restricted to females that were sampled at the same stage of laying in the two successive clutches: plasma VTG levels were highly repeatable within individuals between breeding attempts ($r = 0.93$, $F_{11,12} = 26.2$, $P < 0.001$, $n = 12$, Fig. 2b) and individual explained 92.6% of the total variation in plasma VTG.

**Plasma VTG at the 1-egg stage and reproductive effort**

Body mass at the 1-egg stage, laying interval, total number of days skipped during the laying cycle, and laying rate were independent of diet ($P > 0.05$ in all cases, Table 1). Clutch size, laying interval, total number of days skipped during the laying cycle, and laying rate were also independent of circulating VTG, regardless of diet (ANCOVA controlling for diet, $P > 0.05$ in all cases). In contrast, diet had a significant effect on residual mean egg mass (controlling for body mass, $F_{1,50} = 16.8$, $P < 0.001$), clutch size ($F_{1,50} = 13.1$, $P < 0.001$), and plasma VTG levels ($F_{1,49} = 4.9$, $P < 0.05$; Table 1). Females whose diets were supplemented with egg-food laid larger eggs, more eggs, and had lower plasma VTG than females fed only seed. Furthermore, the relationship between residual mean egg mass and plasma VTG was diet-dependent (ANCOVA controlling for diet; VTG by diet interaction, $F_{1,48} = 10.4$, $P < 0.01$); consequently, the two diets were analysed separately. Residual mean egg mass was negatively correlated with plasma VTG in females supplemented.
Table 1. Variation in body mass, reproductive effort, and plasma VTG in relation to diet in female zebra finches. Plasma VTG was log$_{10}$-transformed for statistical analyses. Values are least-squares means ± SE, with sample sizes in parentheses. Values with the same superscript do not differ significantly (P > 0.05).

<table>
<thead>
<tr>
<th>Trait</th>
<th>Experienced females on seed</th>
<th>Experienced females on seed and egg-food supplement</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body mass (g) at pairing</td>
<td>15.6 ± 0.4 (25)$^a$</td>
<td>16.3 ± 0.3 (25)$^a$</td>
</tr>
<tr>
<td>Mean egg mass (g)</td>
<td>0.995 ± 0.018 (25)$^a$</td>
<td>1.108 ± 0.017 (28)$^b$</td>
</tr>
<tr>
<td>Mass-corrected mean egg mass (g)</td>
<td>−0.051 ± 0.017 (25)$^a$</td>
<td>0.045 ± 0.016 (28)$^b$</td>
</tr>
<tr>
<td>Clutch size</td>
<td>4.0 ± 0.2 (25)$^a$</td>
<td>5.4 ± 0.2 (28)$^b$</td>
</tr>
<tr>
<td>Laying interval (days from pairing)</td>
<td>5.5 ± 0.4 (25)$^a$</td>
<td>5.7 ± 0.4 (28)$^a$</td>
</tr>
<tr>
<td>Plasma VTG (μg/ml, VTG-zinc)</td>
<td>2.04 ± 0.19 (25)$^a$</td>
<td>1.47 ± 0.18 (26)$^b$</td>
</tr>
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</table>

with egg-food ($r = -0.36$, $F_{1,24} = 3.5$, $P = 0.07$), but these traits were positively correlated in the seed-diet females ($r = 0.50$, $F_{1,23} = 7.7$, $P < 0.01$, Fig. 3a). The extreme mean egg sizes in both diet groups (small eggs in seed-diet females, large eggs in females supplemented with egg-food) appear to be driving these relationships between egg size and plasma VTG. Therefore, the relationship between mean egg mass and circulating levels of VTG was assessed using the range of mean egg sizes that were common to both groups (0.913 to 1.154 g). In this restricted analysis plasma VTG levels did not differ between the two diets ($F_{1,36} = 2.8$, $P > 0.10$), and the interaction between plasma VTG levels and diet was not significant ($F_{1,35} = 2.8$, $P > 0.10$). Mean egg mass was not related to plasma VTG levels over this range of egg sizes ($F_{1,36} = 0.3$, $P > 0.50$, Fig. 3b), despite marked (over seven-fold) variation in plasma VTG among individuals.

**Discussion**

In this study we have confirmed that plasma VTG levels are highly variable in female zebra finches during egg-formation, as has been reported previously for the zebra finch (Williams and Christians 1997) and the European starling (Christians and Williams 2001a). However, more importantly we have shown that this individual variation is independent of diet (see Fig. 3) and is non-random: plasma VTG levels vary systematically and predictably through the laying cycle, and plasma VTG is highly repeatable within individuals in successive breeding attempts (i.e. there is little intra-individual variation). While individual variation in the circulating levels of VTG was unrelated to clutch size, laying interval, or laying rate, we have confirmed a complex, diet-dependent relationship between egg size and yolk precursor levels, though mainly through the influence of very small and very large eggs (see below).

**Daily variation in plasma VTG**

In general, the pattern of variation in plasma VTG matched the predicted demand, based on the timing and pattern of follicle development, consistent with the idea that VTG production is costly and that selection has matched supply and demand (sensu Diamond and Hammond 1992). Plasma VTG was not detectable in non-breeding females zebra finches, as was the case with free-living, non-breeding European starlings (Challenger et al. 2001) confirming that production of VTG is tightly coupled to breeding and, specifically, to yolk formation. However, within two days of pairing, but before we predicted that yolk development had started (i.e. >4 days before the first egg was laid), plasma VTG started to increase slightly in zebra finches on different diets (○ seed; ● egg-food supplement) and (b) on different diets and restricting egg size to masses common to both diet groups.
finches, despite the lack of follicle demand for yolk precursors at this time. Redshaw and Follett (1976) reported an initial rise in circulating VTG 4 to 6 weeks prior to onset of laying in domestic hens, even though the duration of RYD in this species is only 8–10 days (Etches 1996). In contrast, circulating levels of VTG in free-living European starlings did not increase until onset of the RYD stage (Challenger et al. 2001). Nevertheless, it is clear that even in zebra finches, highly elevated plasma levels of VTG only occur coincident with the period of rapid yolk development. Moreover, the marked cyclical pattern of ovarian development and yolk precursor production, characteristic of seasonally-breeding birds, is clearly not absent in female zebra finches (cf. Vleck and Priedkalns 1985).

Plasma VTG levels remained elevated throughout the period of maximum follicle demand, from initiation of RYD to the 3-egg stage, as was the case in free-living European starlings (Challenger et al. 2001). In our captive, breeding zebra finches, follicle demand at the 1-egg and 3-egg stages were comparable: the third, fourth, and fifth eggs were undergoing rapid yolk development at the 1-egg stage and the fifth, sixth and possibly seventh eggs were developing at the 3-egg stage (since the mean clutch size of our population was 5.6 ± 2.0 eggs). At the 5-egg stage, plasma VTG levels had started to decline, but were significantly higher than zero, even though it is only in larger clutches (seven eggs or more) that rapid yolk development would still be taking place at the 5-egg stage. Therefore, plasma VTG remained elevated near the end of the laying cycle, when follicle demand was either very low, or non-existent. A possible explanation for maintaining elevated levels of VTG at the 5-egg stage is that a level exists for circulating levels of the yolk precursors below which appropriate levels of receptor-mediated yolk uptake cannot be maintained. Evidence for this interpretation comes from the fact that experimentally decreased plasma VTG levels, through acute and chronic administration of the anti-estrogen, tamoxifen, results in a decrease in the mass and yolk protein content of eggs undergoing RYD during tamoxifen administration (Williams 2000, 2001). Alternatively, the elevated plasma VTG at the end of laying could reflect yet-to-be metabolized VTG particles that were produced earlier to ensure proper development of previous eggs (i.e., fifth and sixth eggs). Redshaw and Follett (1976) suggested that the half-life of VTG in the laying hen is about 1 day. If the half-life of VTG in zebra finches is comparable, then the elevated plasma VTG at the 5-egg stage may be VTG that was previously produced at the 3- and 4-egg stages. In general, however, female zebra finches do appear to match circulating yolk precursor levels to predicted demand from the developing ovarian follicle hierarchy.

**Individual variability and repeatability of plasma VTG**

This study and previous studies on zebra finches and European starlings have found a large degree of inter-individual variation (8- to 10-fold) in plasma VTG levels at the 1-egg stage (Williams and Christians 1997, Christians and Williams 2001a). However, in our study plasma VTG in female zebra finches also exhibited high intra-individual repeatability, that is, individual females had consistently high (or low) circulating levels of VTG during successive breeding bouts. Plasma VTG levels were also shown to be highly repeatable in free-living European starlings, thus suggesting that this is a distinct phenotypic trait (Challenger et al. 2001). Sufficient intra-individual repeatability and inter-individual variation are essential for traits to respond to selection. However, other factors, such as differential fitness exhibited by individuals possessing these traits, and a trait’s heritability and genetic correlations with other traits are also essential for determining the potential of a trait to respond to selection (Stearns 1992). To date there have been no studies examining the heritability of plasma VTG levels in birds. However, Nestor et al. (1996) reported a low heritability estimate ($h^2 = 0.29 ± 0.01$) for total plasma phosphorus (TPP), an index of plasma yolk-targeted very-low density lipoprotein (VLDLy), the other avian egg yolk precursor, in captive Japanese quail Coturnix coturnix japonica selected for increased TPP and body mass. High repeatability of a trait, as we found for VTG, only sets an upper limit on heritability. If plasma VTG actually exhibits low heritability (as for VLDL, Nestor et al. 1996) this would suggest that individual circulating levels of the yolk precursors may be organized early in life, e.g. by environmental conditions experienced during rearing or by maternal effects (Stearns 1992, Bernardo 1996). Future studies should assess effects of rearing condition or maternal effects on reproductive physiology by comparing maternal versus offspring trait values.

**Plasma VTG at the 1-egg stage and reproductive effort**

Christians and Williams (2001a) and Challenger et al. (2001) reported negative relationships between circulating levels of VTG and various measures of reproductive output in European starlings. In contrast, preliminary data for zebra finches suggested that an index of reproductive output (combining total ovary mass,oviduct mass, and mass of the first egg) was independent of plasma VTG (Williams and Christians 1997). The present study has clarified the discrepancy between these results. Female zebra finches on a poor quality seed diet exhibited a weak, positive relationship between circulating VTG levels and egg size. In contrast,
a negative relationship, like those reported for European starlings, was observed between plasma VTG and egg size in females on a higher quality diet (egg-food supplemented). In both cases, females that laid either very small or very large eggs appeared to be driving the relationship (i.e., very small-egg birds and very large-egg birds had low plasma VTG levels). When egg size was limited to the range common to both groups, diet, per se, did not affect plasma VTG levels, as there was no difference in plasma VTG in experienced females on the low and high quality diets. We therefore suggest that the different relationships between VTG and egg mass (one positive, one negative) occurred because the different diets increased the range of egg sizes, and allowed us to detect the effect of very small and very large eggs. Individual females that lay small eggs might do so because they are actually limited by the size of the yolk precursor pool in their plasma, i.e., their circulating levels of VTG may fall below the level required to maintain high levels of receptor-mediated yolk uptake. Again, this would be consistent with experimental evidence that a 30–50% reduction in circulating levels of VTG, caused by administration of the anti-estrogen tamoxifen lead to an 11–15% decrease in egg mass (Williams 2000, 2001). Conversely, in females that produce the largest eggs, yolk formation may actually deplete the plasma VTG pool with uptake of VTG exceeding the rate of VTG synthesis and secretion by the liver. In support of this, yolk mass is positively correlated with rates of yolk precursor uptake in female zebra finches (Christians and Williams 2001b).

Although there is some systematic relationship between circulating levels of VTG and egg or yolk size, this relationship is relatively weak ($r^2 = 10–40\%$; this study; Christians and Williams 2001a, Challenger et al. 2001). Rate of egg-laying was also independent of plasma VTG levels in zebra finches (although Redshaw and Follett (1976) reported that laying hens with irregular laying patterns had higher and more varied concentrations of plasma VTG than hens that laid regularly). Thus, the marked individual variation in circulating VTG levels appears to be a poor indicator of egg production ‘performance’. It is notable too that number and size of eggs laid are independent of marked individual variation in relative liver size (the organ responsible for yolk precursor production), or the relative size of metabolic and digestive organs that would support costs of egg formation (e.g. heart, muscle, gut) in this species (T.D. Williams unpubl. data) and in the European starling (Christians and Williams 2001a). It is possible that individual females must maintain different minimum levels of circulating yolk precursors in order to maintain high yolk uptake rates (e.g. due to individual or follicle-specific differences in VTG-receptor density (Tyler et al. 1990) or follicle vascularisation (Gerrard et al. 1973)). In other words, some females may require a large pool of VTG in order to efficiently produce an average-sized egg, while other females can produce the same-sized egg while maintaining a smaller plasma VTG pool. This difference in ‘efficiency’ of the uptake process could effectively result in the observed large inter-individual variation in circulating levels of VTG (Williams and Christians 1997, Christians and Williams 1999, Challenger et al. 2001). We know that yolk uptake rates vary among individual female zebra finches, and that this variation is repeatable within individuals (Christians and Williams 2001b). However, future studies should address individual variation in other components of the yolk uptake process such as VTG receptor number, internalization rates, and receptor recycling (Shen et al. 1993).

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