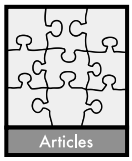

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Pre-breeding diet affects the allocation of yolk hormones in zebra finches *Taeniopygia guttata*

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The ability of mothers to modify offspring phenotype to match prevailing environmental conditions is an important component of reproductive success, especially in variable environments. Pre-breeding conditions, such as food abundance, may have significant consequences for both the number and quality of offspring a female produces as well as her ability to rear the offspring. In an experiment where pre-breeding diet was manipulated, we investigated if allocation of yolk androgens (testosterone and 5α-dihydrotestosterone) was related to the quality of diet experienced prior to breeding. Female zebra finches *Taeniopygia guttata* produced larger clutches on high quality diet than on low quality diet but with no differences in egg mass. Yolk androgen levels were repeatable within subsequent clutches of the same female and females did not change mean androgen content in eggs in relation to diet quality. However, within-in clutch pattern of yolk testosterone and DHT changed with diet treatment. Testosterone and DHT decreased with laying order on the low quality diet but remained constant on the high quality diet. Differential yolk androgen allocation within the clutch may alter the competitive differences between chicks and provide females the possibility to adjust reproductive investment and offspring phenotype already at the egg stage.

Food availability may have substantial effect on the relative costs of reproduction and thus may be an important factor in determining reproductive strategies (Martin 1987). Investment in reproduction is expected to vary according to costs and benefits of raising young. When food resources are abundant, the reproductive value of offspring will be higher due to reduced cost of raising young and females are expected to invest more in reproduction (Hochachka 1992). Studies in which food have been supplemented in the prebreeding season show effects on both timing of reproduction and allocation of resources into the reproductive event (Daan et al. 1988). The availability and quality of food experienced prior to breeding could influence reproductive investment by acting on maternal condition directly or function as a cue to anticipate conditions during later stages of the breeding cycle. When anticipating better conditions or under good breeding conditions, females may increase their investment in reproduction by producing larger clutches (Smith et al. 1993, Houston et al. 1995b), larger eggs (Smith et al. 1993) or increase the quality of the egg. Females may also allocate resources differentially between eggs within the clutch, e.g. in relation to the order in which the eggs are laid (Slagsvold et al. 1984) or the sex of the offspring (Appleby et al. 1997, Rutstein et al. 2005). Differential allocation patterns may reflect female strategies to avoid or facilitate brood reduction (Wiebe 1995).

Females deposit numerous components into eggs which may influence offspring quality, e.g. immunoglobulins (Saino et al. 2002), carotenoids (Blount et al. 2002, Royle et al. 2003) and steroid hormones (Schwabl 1993). Such early maternal investments can increase reproductive success by adaptively influence chick phenotype (Rossiter et al. 1996, Mousseau and Fox 1998). Increased concentrations of yolk steroids is thought to produce phenotypic effects that enhance the competitive ability in offspring (Lipar et al. 1999, Eising et al. 2001), although increased levels of yolk

androgens have also been reported to have negative effects (Sockman and Schwabl 2000). Female allocation of yolk androgens may represent a form of maternal investment in offspring, particularly if the levels are related to the quality of the female or her mate (Gil et al. 1999, Pilz et al. 2003). Intraspecific variation in yolk androgen allocation is also related to variation in maternal social environment (Whittingham and Schwabl 2002, Mazuc et al. 2003) and breeding conditions, such as day length or breeding density (Schwabl 1993, 1997). Within-clutch differences in yolk steroid may be adaptive in asynchronously hatching species since its effect on offspring competitive ability enhance or mitigate sibling interactions (Eising et al. 2001).

Zebra finches *Taeniopygia guttata* live in the semi-arid regions of Australia and are considered to be opportunistic breeders based on their ability to nest immediately in response to changes in environmental conditions (Zann 1996). Any further ability to optimise the reproductive effort in relation to the quality or quantity of food resources would be highly adaptive. Zebra finches are protein-limited during egg production (Houston et al. 1995b, Williams 1996). Experiments with captive zebra finches have shown that females on a protein-rich diet have larger clutches and heavier eggs (Selman and Houston 1996, Monaghan et al. 1996, Williams 1996, Rutstein et al. 2004) as well as greater hatching success (Selman and Houston 1996, Gorman and Nager 2003).

In this study, we experimentally manipulated nutritional quality of the diet prior to breeding in captive zebra finches and analysed female allocation of maternal steroid hormones into egg yolk. We predicted that the allocation of yolk androgens would differ in relation to diet quality, either by variation in total allocation of yolk androgens or as a change in within-clutch pattern of yolk androgens.

Methods

We used wildtype, out bred birds from two different captive populations. Age varied between eight months and two years and the birds had no previous breeding experience. They were maintained at a temperature of 19–23°C and a constant lighting schedule of 14 h light: 10 h dark. Before the start of the diet manipulation, males and females were kept separated in single-sex cages in audiovisual contact with birds of the other sex. Water, cuttlebones and *Panicum* millet seeds were available *ad libitum*. Greenery was provided two times per week. At the start of the diet experiment, females were randomly paired with a male. Each pair was placed in a breeding cage (60 cm wide × 35 cm deep × 39 cm high).

Pre-breeding diet was manipulated for three weeks prior to egg-laying by giving the birds two different diets. Several studies have manipulated zebra finch diet prior or during breeding by giving extra protein and/or lipids, mainly by adding albumin, yolk or whole hard-boiled chicken eggs (Selman and Houston 1996, Williams 1996, Williams and Matryniuk 2000, Rutstein et al. 2005). We decided to use the diet treatments used in Williams (1996) and Williams and Matryniuk (2000) and provided whole hard-boiled chicken eggs. Low quality diet (LQD) consisted of the same diet as before the start of the experiment with *ad libitum* panicum millet seeds (12% protein). Birds on the high quality diet (HQD) received a mixture of *ad libitum* yellow, red and white millets (*Setaria* and *Panicum* ssp.) with canary grass seeds, and oat groats (14% protein) together with a daily supply of hard-boiled hen's egg (10 grams mixture of yolk and albumin/day/pair, 20,3% protein and 6.6% lipids).

The pairs was divided into two equal sized groups and given either LQD or HQD. After three weeks, both groups were given *ad libitum* panicum millet seeds. Each breeding cage was at the same time provided with nest box and nesting material. Nest boxes were checked daily and all new eggs taken from the nest and replaced with plastic eggs. All eggs were weighed, individually marked and frozen at −20°C. Three to five days after the clutch was completed we removed the nest box with the plastic eggs. To enable paired comparisons of individual females, the pairs were allowed a second breeding attempt on the opposite diet. The pairs were fed the opposite diet for three weeks starting seven days after clutch completion. The experimental setup was then identical to the first breeding attempt. We replaced eggs with dummies on the day of laying and terminated the experiment three days after the last egg was laid.

Female tarsus length was measured at the start of the experiment and we measured female body mass three times during each treatment, at the beginning of the diet manipulation period, at the time of nest box provisioning and after the clutch was completed.

Hormone analyses

Shell, albumin and yolk were separated during thawing of the egg to allow for analyses of albumin and yolk mass. After yolk mass was measured, the yolk was homogenized and a small sample (approx. 25 mg) was used for hormone analysis. Individual samples were mixed with 500 µl distilled water in 1.5 ml Epindorf tubes using a Vortex mixer. Homogenization was facilitated by the addition of a few glass beads in each tube (Schwabl 1993).

All samples were analyzed for the presence of testosterone (T) and 5α-dihydrotestosterone (DHT)

with radioimmunoassay as outlined by Wingfield and Farner (1975) and modified by Schwabl (1993). Tritiated forms of each steroid were added to each sample for calculations of recovery percentages following extracting and chromatography (approx. 2000 cpm, Perker Elmer Life Sciences). Steroids were extracted twice with 3 ml petroleum and diethyl ether (30:70%), following a precipitation with 95% ethanol to remove excess proteins and lipids. The samples were resuspended in re-distilled ethyl acetetate and iso-octane (10:90%). The extracts were applied to chromatography columns with a celite: ethylene glycol: propylene glycol (6:1.5:1.5, w:v) upper phase and a celite: water (3:1, w, v) lower phase. DHT and T were collected from the phases eluded with 10% and 20% re-distilled ethyl acetate in iso-octane, respectively.

After separation, hormones were measured by radioimmunoassay with tritiated hormone and antibodies (Wien Laboratories and Research Diagnostics Inc). Each sample was run in duplicates and mean values were compared with a standard curve (1.95–500 pg/sample). Samples were run in two assays, with the restriction that eggs from individual females were processed in one assay and were not divided across assays. Intra-assay coefficient of variation, based on standard samples of known concentrations, ($n = 6$ in each assay), was 1.8 for testosterone and 12.6% for 5α-dihydrotestosterone in the first assay and 8.9% (testosterone) and 12.8% (DHT) in the second assay. Interassay variation was 5.9% for testosterone and 1.5% for 5α-dihydrotestosterone. Recoveries ranged from 43–75% (mean 56%).

We report hormone content as both yolk concentration (pg/mg yolk) and as total yolk hormone content (pg/whole yolk), as the amount of hormone that an embryo is exposed to will vary with both yolk mass and yolk concentration. Work was carried out under the licence from the Animal Care and Use Committee at Cornell University.

Statistical analyses was conducted with SAS System for Windows (version 9.1). Yolk androgen levels and egg mass was not normally distributed but $\log_{10}$ transformation successfully normalized data. Paired t-test was used to analyse differences in mean egg mass and mean hormone content between treatments. We used general linear mixed models (PROC MIXED, Littell et al. 2004) for analyses of within-clutch patterns of yolk hormones and egg mass. Diet treatment was used as a fixed factor and laying order as a covariate and brood nested within female as subject. Female identity together with its interaction with the fixed factor and covariate were included as a random factor in the model. The random effect was estimated with the likelihood ratio test (Little et al. 2004). We used the Satterwaithe method to estimate the denominator degrees of freedom (ddf) which perform well with unbalanced data sets (Littell et al. 2004). We also included clutch order (first or second clutch) as well as order of treatments in all models to test for carry-over effects but none of the two variables or their interactions with other variables were significant ($P > 0.20$) and thus we dropped the variables from the models. Clutch size and timing of breeding could not be normalized and non-parametric paired tests were used. All tests were two-sided and conducted at the 5% significance level.

Results

Two females were excluded from all analyses because they did not breed during either experiment. Two females bred during only one of the treatments ($n = 1$ during LQD and $n = 1$ during HQD) and they were included in all analyses except pair-wise comparisons. There was no difference in days between nest supply and start of egg laying between clutches on HQD and LQD (Wilcoxon signed rank test, $n = 12$, $P = 0.58$), or between first and second clutch ($n = 12$, $P = 0.09$).

Egg mass and egg components

Egg mass varied between 0.80 and 1.21 g (mean 1.03 ± 0.08 g) and increased with laying order ($F_{1,12.5} = 17.44$, $P = 0.001$). There was a significantly between-female difference in egg mass ($\chi^2 = 10$, $df = 1$, $P = 0.001$), and mean egg mass was highly repeatable within individual females between clutches ($r = 0.71$, $F_{11,12} = 6.06$, $P = 0.002$). However, pre-breeding diet did not influence mean egg mass ($F_{1,20.3} = 0.01$, $P = 0.94$), or within-clutch patterns of egg mass (the interaction between laying order and diet, ($F_{1,17.1} = 0.12$, $P = 0.73$). Yolk mass correlated with egg mass (Pearson' correlation, $r = 0.71$, $n = 107$, $P < 0.01$) but large eggs did not contain disproportionately more yolk (log-log regression, following Ricklefs et al. 1978, $b = 1.02$, $SE = 0.098$). Diet did not affect the ratio of yolk: albumin (clutch mean, $t = -0.81$, $n = 10$, $P = 0.43$).

Effects on clutch size and female condition

Clutch size varied between 3 and 7 eggs and females laid larger clutches on HQD than on LQD (4.92 ± 1.24 compared to 4.17 ± 1.15 eggs, Wilcoxon rank test, $n = 12$, $P = 0.021$). Females increased in body mass during the pre-breeding period, with on average 7.4% (total body mass) whereas body mass decreased during egg laying period (with on average 6.7% of total body mass). There was no effect on pre-breeding diet on body mass increase before laying, (paired ttest, pre-breeding period,

$t = 0.57$, $n = 11$, $P = 0.58$) or mass decrease during egg laying ($t = -0.67$, $n = 11$, $P = 0.52$).

Yolk hormones

Testosterone concentration in egg yolk ranged from 2.0 to 36. 9 ng/mg yolk (mean 13.6 ± 7.4 ng/mg yolk) and DHT concentration ranged from 2. 7 to 35.2 ng/mg yolk (mean 12.9 ± 6.0). DHT levels were positively correlated with testosterone levels ($r = 0.64$, $F_{1,105} = 70.8$, $P < 0.001$) indicating that females that allocated more T to eggs also allocated more DHT. Total yolk testosterone per egg varied between 46.16 and 851.08 ng (mean 330.84 ± 187.27 ng). DHT levels were similar in magnitude and variability (range 63.72–916.36, mean 312.92 ± 152.23 ng/egg), and positively correlated to yolk testosterone ($r = 0.65$, $F_{1,105} = 77.9$, $P < 0.001$).

Females did not vary clutch mean of yolk testosterone concentration and yolk DHT concentration when fed low versus high quality diet prior to egg-laying (paired ttest, testosterone, $df = 11$, $P = 0.76$, DHT $P = 0.79$). However, within-clutch variation of yolk testosterone was related to pre-breeding diet, as revealed by a significant interaction between diet and laying order ($F_{1,13} = 11.53$, $P = 0.005$, Fig. 1). Testosterone concentration decreased significantly with laying order when the females were on LQD ($F_{1,8.97} = 16.42$, $P = 0.003$) but there was no laying order effect in testosterone concentration when females was on HQD ($F_{1,11.1} = 0.55$, $P = 0.47$). Total yolk testosterone showed a similar interaction between laying order and diet ($F_{1,12.7} = 4.72$, $P = 0.049$). This pattern was also found in DHT concentration, ($F_{1,47.4} = 5.27$, $P = 0.027$) or yolk DHT ($F_{1,26.5} = 5.88$, $P = 0.022$).

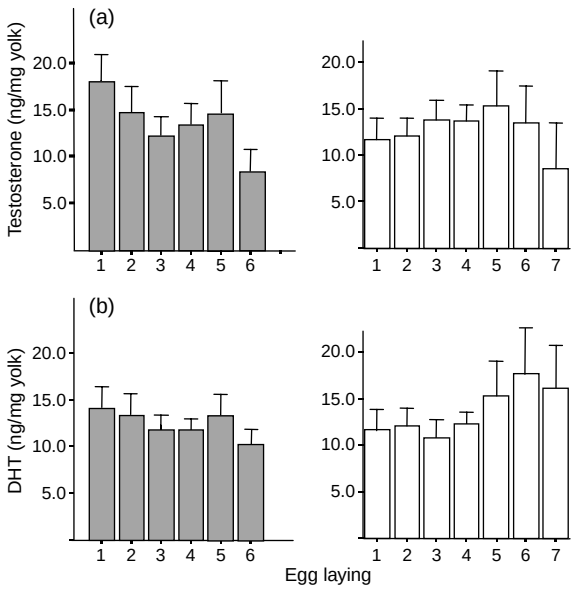

Fig. 1. Within-clutch patterns of yolk androgen concentration in eggs of female zebra finches on a prebreeding diet of low (grey bars) vs high quality (open bars). (a) yolk testosterone (ng/mg yolk), and (b) yolk DHT concentration (ng/mg yolk). Mean ± SE.

There was a significant between-female variation in both testosterone and DHT concentration (testosterone, $\chi^2 = 4.9$, $d = 1$, $P = 0.013$, and DHT $\chi^2 = 6.5$, $df = 1$, $P = 0.005$), and total amount of yolk testosterone and DHT (testosterone $\chi^2 = 8.4$, $df = 1$, $P = 0.002$, DHT: $\chi^2 = 12.2$, $df = 1$, $P < 0.001$). Mean hormone concentration was also highly repeatable within individual females between their first and second clutch (testosterone $r = 0.64$, $F_{11,12} = 4.56$, $P = 0.007$, and DHT, $r = 0.68$, $F_{11,12} = 5.09$, $P = 0.005$, Fig. 2).

Discussion

Allocation of androgens to egg yolk was influenced by the quality of the pre-breeding diet. Average level of yolk androgens did not differ between treatments but females changed the within-pattern of yolk hormones deposition in relation to diet quality. Testosterone concentration in yolk decreased with laying order on the low quality diet but remained constant with respect to laying order on the high quality diet. DHT showed the same pattern. In addition, females on a high quality diet laid larger clutches than females on a low quality diet.

Zebra finches are considered as capital breeders which is unusual among small passerines and females rely partly on body reserve nutrients for reproduction (Houston et al. 1995a). In captive as well as in wild populations, female zebra finches increase in body mass prior to breeding (Williams and Ternan 1999, Rozman et al. 2003). In this study, we increased the quality of pre-breeding diet by adding hardboiled hen's egg and providing a wider array of seed. Hen's egg contains a majority of the nutrients used in egg production, e.g. proteins, lipids and carotenoids. Williams (1996) showed that only supplementation of proteins, not lipids, had a positive effect on reproductive output in zebra finches. Together with the fact that zebra finches use endogenous protein for egg production (Houston et al. 1995b, Cottam et al. 2002), we suggest that protein was an important component of the experimental diet but can not exclude the importance of other components.

Effects of within-clutch patterns of yolk hormones

Individual females showed a capacity to vary the allocation of yolk hormones within the clutch depending on the quality of the diet they experienced before breeding. When fed a low protein diet, females

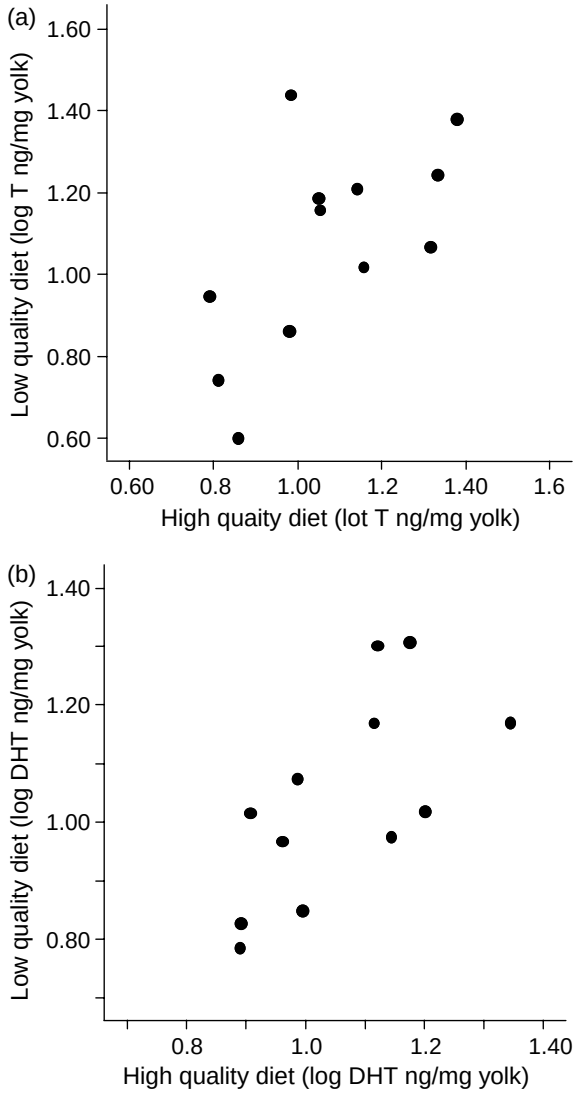

Fig. 2. Mean yolk androgen concentration in eggs from individual females on low and high quality diet, Fig. 2a shows testosterone concentration (ng T/mg yolk), and Fig. 2b DHT concentration (ng/mg yolk).

decreased testosterone and DHT levels in later laid eggs compared to a more equal deposit of yolk hormones over the laying sequence on a high protein diet. The adaptive benefits of differential allocation of yolk steroids within the clutch have often been related to sibling competition and hatching asynchrony. One group of hypotheses relates the benefits of hatching asynchrony to the production of a size hierarchy. The size hierarchy that arises among chicks of a brood when the parents begin to feed the first-hatched young before the last-hatched young are present produces a competitive disadvantage for the late-hatched young. When food condition during nestling rearing is poor, the established size hierarchy would facilitate brood reduction if parents focus their attention on the larger young, which they typically do (Ostreiher 1997, Cotton et al. 1999).

Individuals may vary the degree of hatching asynchrony in relation to environmental quality and hence, manipulate the competitive variation within a brood, (Magrath 1989, Wiebe and Bortolotti 1994, 1995) If food resources during breeding are partly predicted from those during the pre-breeding period, theoretical models show that facultative hatching asynchrony may be adaptive (Wiebe 1995). Differential allocation of yolk androgens within the clutch with decreased androgen levels in later laid eggs may enhance the effect of brood reduction and bias investment towards offspring of higher reproductive value. Zebra finches are known to hatch asynchronously. Zann (1996) found that in wild zebra finches, brood reduction is common with majority of nestling mortality among the smallest individuals. Zebra finches may use within-clutch variation in yolk androgens to vary the level or the timing of brood reduction in relation to expected environmental conditions during nestling rearing or to their own rearing capacity. Females adopt a brood reduction strategy on low quality diet but a brood survival strategy on high quality diet (*sensu* Schwabl 1997, Gil 2003). The same mechanism has been proposed for the allocation of antioxidants in eggs (Royle et al. 2003).

Effect on female patterns of yolk hormones

In this study, a large proportion of the variation in yolk hormones is due to differences between females. Several other species also show large inter-individual differences in yolk androgens (Schwabl 1996, Lipar et al. 1999, Reed and Vleck 2001, Groothuis and Schwabl 2002, Pilz et al. 2003). However, this study also shows that yolk hormone levels are highly repeatable among clutches of the same female even if females change within-clutch patterns. Individual females that had relatively high levels on high quality diet also had high levels when fed a low quality diet. If there is a genetic basis to variation in yolk hormones, maternal allocation of yolk hormones is an indirect genetic effect with important evolutionary consequences (Mousseau and Fox 1998).

Non-adaptive explanations

Differential allocation of yolk hormones may also have non-adaptive explanations. If females are depleted of nutrients essential for hormone production on a low quality diet, hormone levels may decrease with laying order. However, clutch mean of yolk hormones did not differ between treatments suggesting that females did

not experience depletion of hormones with laying order on a low quality diet. Another possibility is that differences in yolk androgen levels are non-adaptive correlations with physiological processes within the female. There are a few studies that shown a correlation between androgen level in female plasma level and yolk androgen levels (Schwabl 1993, Mazuc et al. 2003). If female plasma levels would vary with differences in the breeding environment, the allocation pattern to yolk found in this and other studies could simply reflect female androgen levels and not directed allocation to eggs. There is a need for more studies on the relationship between plasma hormones in the adult female, their deposition in the egg and the potential trade-off between optimal levels of androgens in females and eggs.

Effect on clutch size and egg size

Females on a high quality diet laid larger clutches than females on a low quality diet as has been shown by several other studies (Monaghan et al. 1996, Selman and Houston 1996, Williams 1996). Egg size is generally less influenced by food conditions than clutch size, and egg size variation is typically maintained even on supplemental diets of high quality (Christians 2002), but protein supplementation during the pre-breeding (Selman and Houston 1996, Rutkowska and Cichon 2002) and egg formation (Williams 1996) has been shown to increase egg size in zebra finches. However, in this study, we found no effect of pre-breeding diet on clutch egg mass or on the ratio of yolk: albumin.

In conclusion, female zebra finches varied the number of young and their potential competitive ability mediated by hormones in relation to food quality during the pre-laying period. Whether this is an adaptive response that adjusts maternal investment to rearing conditions likely to be encountered during the nestling phase or a consequence of female condition or both cannot be determined in this study. But it is important to recognize that females have the possibility to adjust reproductive investment and offspring phenotype already before egg laying. Such maternal effects can have an important role in fine-tuning reproduction not only in variable environments.

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