LETTER

Natural selection acts in opposite ways on correlated hormonal mediators of prenatal maternal effects in a wild bird population

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Abstract

Maternal hormones are important mediators of prenatal maternal effects. Although many experimental studies have demonstrated their potency in shaping offspring phenotypes, we know remarkably little about their adaptive value. Using long-term data on a wild collared flycatcher (*Ficedula albicollis*) population, we show that natural selection acts in opposite ways on two maternally derived androgens, yolk androstenedione (A4) and yolk testosterone (T). High yolk A4 concentrations are associated with higher fitness, whereas high yolk T concentrations are associated with lower fitness. Natural selection thus favours females that produce eggs with high A4 and low T concentrations. Importantly, however, there exists a positive (non-genetic) correlation between A4 and T, which suggests that females are limited in their ability to reach this adaptive optimum. Thereby, these results provide strong evidence for an adaptive value of differential maternal androgen deposition, and a mechanistic explanation for the maintenance of variation in maternal investment in the wild.

Keywords

Adaptation, adaptive phenotypic plasticity, maintenance of variation, maternal effects, natural selection, prenatal effects, trade-off, transgenerational effects, yolk androgens, yolk hormones.

INTRODUCTION

Adaptive phenotypic plasticity allows individuals to modify their morphology, physiology and behaviour in response to environmental cues to maximise their fitness (Pigliucci 2001). Transgenerational effects are a special case of phenotypic plasticity, in which the cues that affect trait expression are produced or modified by the parents (Mousseau & Fox 1998). Indeed, in most species, it is the mother who provides the first environment an individual encounters in its life, even before it is born (Mousseau & Fox 1998). Because phenotypic plasticity is not unlimited (DeWitt *et al.* 1998), and many fundamental aspects of body organisation and functioning are canalised early in life (Morgane *et al.* 1993; Balthazart & Adkins-Regan 2002), such prenatal maternal effects are assumed to have long-lasting fitness consequences (Rossiter 1996; Mousseau & Fox 1998).

Important mediators of prenatal maternal effects are hormones that are transferred from the mother to the offspring during pregnancy (Weinstock 2008) or egg production (Schwabl 1993). In oviparous species, it is particularly maternal androgens, such as testosterone (T) and its precursor androstenedione (A4), that have received considerable attention over the last 20 years (Groothuis *et al.* 2005b; Gil

2008). Experimental studies have found that high testosterone concentrations in the eggs can boost offspring post-hatching growth and competitiveness (Groothuis *et al.* 2005b; Gil 2008; but see Sockman & Schwabl 2000; Pitala *et al.* 2009), which is generally assumed to be beneficial for both maternal and offspring fitness (Tinbergen & Boerlijst 1990; Lindén *et al.* 1992).

These positive short-term effects have raised the question why not all females deposit high yolk androgen concentrations in their eggs, or in other words, how the large variation in maternal hormone deposition typically observed within populations (Groothuis *et al.* 2005b) is maintained. In an attempt to answer this, and the general and enigmatic question of how variation in fitness-related traits is maintained in nature, studies have focused on potential costs of exposure to high yolk androgen concentrations for the offspring, for example, in the form of increased energy expenditure (Tobler *et al.* 2007) or reduced immunocompetence (Groothuis *et al.* 2005a), that might contribute to the maintenance of variation in maternal hormone deposition. However, evidence for such trade-offs is mixed (Eising *et al.* 2003; Tschirren *et al.* 2005; Navara *et al.* 2006). Alternatively, the deposition of high concentrations of maternal androgens might be beneficial for the offspring, but costly for the mother. However, assessing a

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mother's costs associated with high yolk androgen deposition is notoriously difficult (Groothuis & Schwabl 2008).

Thus, whereas experimental work over the last two decades has revealed that maternal hormones are important mediators of transgenerational phenotypic plasticity (Groothuis *et al.* 2005b; Gil 2008), we still know remarkably little about the selective pressures as well as the trade-offs and constraints that shape the evolution and evolutionary consequences of hormone-mediated maternal effects in the wild.

Here, we use data on yolk androgen concentrations, lifetime reproductive success (LRS), lifespan and fecundity from a long-term study population of collared flycatchers (*Ficedula albicollis*) breeding on the Swedish island of Gotland to quantify natural selection acting on maternal yolk androgens via different fitness components. Thereby, our study provides the first estimates of the strength and direction of natural selection acting on maternal yolk hormone deposition, and reveals how constraints contribute to the maintenance of different maternal strategies.

MATERIAL AND METHODS

Study population

The collared flycatcher (*F. albicollis*) study population breeding on the Swedish island of Gotland (57°10′ N, 18°20′ E) has been extensively studied since 1980, and detailed information on reproductive success and lifespan are available for most individuals breeding in the population (Gustafsson 1989). All nestlings are ringed before fledging, and adults are caught at the nest during incubation and/or nestling feeding for identification. Detailed information on the study population and routine field procedures are described in Gustafsson (1989), Pärt & Gustafsson (1989) and Doligez *et al.* (2009).

Egg collection and yolk hormone analysis

Eggs were collected in 2003 ($N = 79$), 2005 ($N = 120$), 2006 ($N = 108$) and 2007 ($N = 39$). At the beginning of each breeding season, we visited the nest boxes to monitor nest building and egg-laying activity. When two eggs were found in the nest, we marked them with a non-toxic marker. On the next day, we returned to the nest box to collect the third egg on the day it was laid. On the same day, the yolk of the collected egg was separated from the albumen and frozen at $-18°C$ for later hormone analysis. Collared flycatchers lay one egg per day, and during the study period 94% of the females laid a clutch of 5–7 eggs. For the great majority of females, the third egg is thus one of the middle eggs of a clutch. Whereas yolk hormone concentrations of the middle egg are representative for the average hormone concentrations nestlings of a brood are exposed during development (Michl *et al.* 2005; Hegyi *et al.* 2011), intra-clutch variation in yolk hormone concentrations (Michl *et al.* 2005; Hegyi *et al.* 2011) will add noise to the data.

In total, eggs of 256 females were analysed for yolk androstenedione (yolk A4) and yolk testosterone (yolk T) concentrations using radioimmunoassay technique as described in Tschirren *et al.* (2009). We corrected the measured androgen concentrations (pg/mg yolk) for extraction efficiency (mean recovery rate: 73%, range 62–83%). Dilution curves confirmed the reliability of extraction and assay protocols. The cross-reactivity of the T antibody is 5.8% for 5α-dihydrotestosterone (DHT) and 2.3% for A4. The cross-reactivity of the A4 antibody with other steroids is less than 1% (Diagnostic Systems Laboratories, Webster, USA). We included duplicates of pooled collared flycatcher yolk samples in each assay to calculate intra- and inter-assay variation. Intra-assay variation was 7.5% for A4 and 6.3% for T. Inter-assay variation was 5.9% for A4 and 7.7% for T.

Yolk androgen concentrations are significantly repeatable within females among years (Tschirren *et al.* 2009). If the same female was sampled in more than 1 year, we therefore used average yolk A4 and yolk T concentrations in the analyses. Yolk A4 and yolk T concentrations were log transformed for the statistical analyses.

Fitness measures

To quantify selection pressures acting on yolk androgen deposition, we used LRS as a measure of fitness, as well as two of its components, namely female lifespan and female fecundity.

Female LRS was measured as the total number of a female's offspring that returned as breeders to the local population (recruits). Recruits were identified when they were incubating eggs or feeding their brood. We analysed lifetime number of recruits produced by a female, and, by including the number of breeding events as a covariate in the model, average annual reproductive success of a female. Furthermore, we determined the number of male and female recruits produced to test for sex-specific associations between yolk androgens and recruitment.

Female lifespan was defined as the last year a female was seen in the study population minus her year of birth. The exact year of birth was known for 127 females (ringed as nestling in the study population). The age of 129 previously unringed females was determined based on plumage characteristics (Svensson 1992). Eighty-one females were 1 year old at first capture, and 48 females were older than 1 year at first capture. For the latter, we assumed that they were 2 years old at first capture. The lifespan of this latter group is thus a minimal estimate. Associations between yolk androgen concentrations and lifespan were similar in females for which the exact age was or was not known (results not shown).

To obtain a measure of female fecundity that is independent of both female lifespan and offspring survival, we calculated for each female the mean clutch size produced over all breeding events.

Breeding data from the study population were available until 2011. Only one female for which we had information on egg androgen concentrations was still observed as a breeder in 2011, and two females were last seen in 2010. All other females were last observed between 2003 and 2009. Nevertheless, to account for the possibility that LRS and/or lifespan might be underestimated in females that were still breeding in more recent years, we included the year a female was last seen in the population as a covariate in the statistical analyses.

Statistical analyses

We calculated a Pearson correlation coefficient to describe the relationship between the two androgens within eggs across females. We then used general and generalised linear models to test for a relationship between yolk androgen concentrations and the three fitness measures outlined above. LRS was analysed with a negative binomial regression using the package MASS (Venables & Ripley 2002), both with and without including the number of lifetime breeding events of the female as a covariate. Female lifespan was analysed using a zero-truncated Poisson regression in the package VGAM (Yee 2013). Residual lifespan was not overdispersed. Female fecundity was analysed using a linear model. Residual fecundity was normally distributed.

To test for sex-specific effects of yolk androgens on the recruitment of male and female offspring, we first fitted a negative binomial mixed model to sex-specific LRS using the package glmmADMB (Skaug *et al.* 2013), including offspring sex, androgen concentrations and their interactions as a fixed effects, and female identity as a random effect. Because there was evidence that yolk A4 concentrations affect local recruitment of male and female offspring in different ways (see Results), we subsequently analysed recruitment of males and females separately using negative binomial regressions.

To obtain a formal estimate of the direction and strength of selection acting on yolk androgen concentrations, we standardised yolk A4 and yolk T concentrations to have a mean of zero and a standard deviation of one, and we calculated relative fitness (i.e. relative number of recruits, relative lifespan and relative fecundity) for each female by dividing number of recruits, lifespan and fecundity by the population mean. We then fitted a linear regression model that included standardised yolk A4 and yolk T concentrations for the estimation of standardised directional selection gradients (β), and a quadratic regression model that included standardised yolk A4 and yolk T concentrations and their quadratic terms for the estimation of quadratic selection gradients (γ) (Lande & Arnold 1983). Quadratic regression coefficients were doubled to obtain estimates of γ (Stinchcombe *et al.* 2008). The interaction term

between A4 and T was included to estimate correlational selection (Blows & Brooks 2003).

Using yolk A4 and yolk T contents rather than yolk A4 and yolk T concentrations in the analyses gave comparable results (results not shown). All analyses were performed in R 2.14.2.

RESULTS

Within-egg relationship between yolk androgens

Yolk A4 and yolk T concentrations were significantly positively correlated within eggs ($r = 0.305$, $P < 0.001$, $N = 256$).
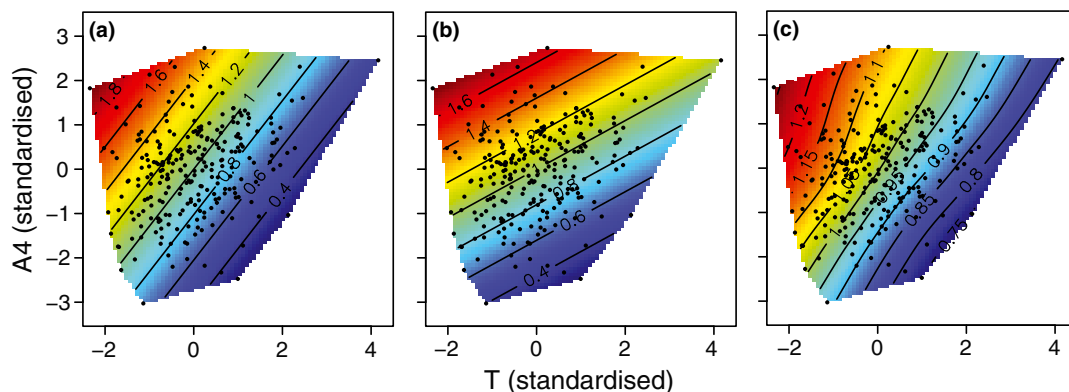
Lifetime reproductive success

Females produced on average (mean $\pm$ 1 SD) 1.2 ± 1.4 recruits (median: 1 recruit; interquartile range: 0–2 recruits; maximum: eight recruits) during their life. Females that deposited higher concentrations of yolk A4 in their eggs produced more recruits ($\beta = 0.195$; $\chi^2 = 7.270$, $P = 0.007$; Fig. 1a), whereas females that deposited higher concentrations of yolk T in their eggs produced fewer recruits ($\beta = -0.251$; $\chi^2 = 11.480$, $P < 0.001$; Fig. 1a). The interaction effect between yolk A4 and yolk T ($\gamma = 0.110$; $\chi^2 = 2.481$, $P = 0.115$) and the quadratic terms of yolk A4 ($\gamma = -0.093$; $\chi^2 = 1.342$, $P = 0.247$) and yolk T ($\gamma = 0.070$; $\chi^2 = 0.240$, $P = 0.624$) were non-significant.

When controlling for the number of breeding events a female had during her life, the positive association between yolk A4 and reproductive success remained strong and significant ($\beta = 0.249$; $\chi^2 = 6.340$, $P = 0.012$; Fig. 1b), whereas the negative association between yolk T and the number of recruits became weaker and no longer reached significance ($\beta = -0.137$; $\chi^2 = 2.922$, $P = 0.087$; Fig. 1b).

Female lifespan

Females lived on average (mean $\pm$ 1 SD) 3.1 ± 1.6 years (median: 3 years; interquartile range: 2–4 years; maximum: 8 years). Females that deposited higher concentrations of yolk

Figure 1 The relationship between yolk androgens and fitness in collared flycatchers. Fitness surfaces (thin plate splines) illustrating the relationship between yolk androgen concentrations (yolk A4 and yolk T) and (a) lifetime reproductive success (total recruits produced), (b) annual reproductive success, and (c) female lifespan. Blue indicates low fitness and red indicates high fitness. Standardised hormone concentrations and relative fitness are shown.

A4 (β = 0.058; z = 1.993, P = 0.046) and lower concentrations of yolk T (β = −0.087; z = −2.588, P = 0.010) in their eggs had a longer lifespan (Fig. 1c). The interaction effect between yolk A4 and yolk T (γ = 0.054; z = 1.075, P = 0.282) and the quadratic terms of yolk A4 (γ = −0.064; z = −0.469, P = 0.639) and yolk T (γ = 0.040; z = 0.378, P = 0.705) were non-significant.

Female fecundity

The average clutch size (mean ± 1 SD) was 6.1 ± 0.7 eggs. There was no significant association between yolk A4 (β = 0.004; $F_{1,251}$ = 0.400, P = 0.528) or yolk T concentrations (β = −0.006; $F_{1,251}$ = 0.875, P = 0.351) and clutch size. The interaction effect between yolk A4 and yolk T (γ = −0.008; $F_{1,248}$ = 0.040, P = 0.841) and the quadratic terms of yolk A4 (−0.789 ± 0.486, $F_{1,248}$ = 2.634, P = 0.106; γ = −0.175) and yolk T (γ = −0.059; $F_{1,248}$ = 0.271, P = 0.603) were non-significant.

Sex-specific effects on recruitment

Yolk A4 had sex-specific effects on recruitment (interaction yolk A4 x sex: z = −2.09, P = 0.037), whereas the association between yolk T and recruitment was similar in the two sexes (interaction yolk T x sex: z = 1.20, P = 0.231).

When analysing male and female offspring separately, we found that the positive association between yolk A4 concentrations and recruitment was pronounced in female offspring (β = 0.296; χ^2 = 11.403, P < 0.001), but absent in male offspring (β = 0.067; χ^2 = 0.486, P = 0.486). High yolk T concentrations in the eggs, on the other hand, were negatively associated with recruitment in both sexes (females: β = −0.293; χ^2 = 11.182, P < 0.001; males: β = −0.197; χ^2 = 4.000, P = 0.046). Nonlinear selection on yolk androgens was non-significant in both males (A4: γ = −0.094; χ^2 = 0.510, P = 0.475; T: γ = 0.060; χ^2 = 0.063, P = 0.802) and females (A4: γ = −0.092; χ^2 = 1.473, P = 0.225; T: γ = 0.078; χ^2 = 0.231, P = 0.631).

In all cases, the androgen concentration associated with maximum fitness was located well outside the observed range of concentrations.

DISCUSSION

We show that natural selection shapes the deposition of two correlated maternal yolk androgens in opposite ways. Using LRS (i.e. the number of offspring of a female that recruited into the breeding population) as fitness measure, we observed directional selection for high yolk A4 concentrations. At the same time, we observed directional selection for low yolk T concentrations. Although the same pattern was found when analysing female lifespan, the higher LRS of females that produced eggs with high yolk A4 and low yolk T concentrations was not just due to the larger number of breeding events of longer lived females. Furthermore, it was not due to these females laying larger clutches. It shows that differences in offspring survival, mediated either through direct effects of yolk androgens on offspring viabil-

ity or indirect effects of yolk androgen deposition on maternal provisioning after hatching, contributed to variation in LRS. Although it is difficult to fully disentangle maternal and offspring fitness (Wolf & Wade 2001), it illustrates that yolk androgens affect different fitness components in consistent ways.

Given the opposite effects of yolk A4 and yolk T on fitness, mothers should produce eggs with high A4 and low T concentrations to maximise their fitness. However, yolk A4 and yolk T were positively correlated within eggs, a pattern that is generally found in birds, both at the within-clutch and among-clutch level (Groothuis & Schwabl 2002; Gil et al. 2004; Postma et al. 2014). It suggests that females are limited in their ability to reach this adaptive optimum.

This positive association between yolk A4 and yolk T is not due to a genetic constraint, because whereas yolk T deposition is heritable, yolk A4 deposition does, at least in this population, not have a heritable basis (Tschirren et al. 2009). Instead, it indicates that physiological constraints prevent an independent transfer of A4 and T to the eggs. Indeed, A4 is the precursor of T, and limitations in the plasticity of the enzymatic pathway that catalyses the conversion of A4 to T may contribute to their correlated deposition (Groothuis & Schwabl 2008).

Since the discovery of yolk hormones in bird eggs two decades ago (Schwabl 1993), numerous studies have investigated their role in mediating (adaptive) transgenerational plasticity (Schwabl et al. 1997; Groothuis et al. 2005b; Gil 2008). However, the large majority of studies have measured or experimentally manipulated yolk T, whereas yolk A4 has received much less attention (Groothuis et al. 2005b; Gil 2008). This focus on T is likely due to its assumed higher biological potency (Groothuis & Schwabl 2008). Our study reveals that yolk A4 and yolk T are associated with different biological consequences (see also Hegyi et al. 2011; Muriel et al. 2013), and that yolk A4 is an equally important, or even more important target of natural selection than yolk T. However, further studies are required to show if selection is acting directly on A4 and T, their metabolites (e.g. estradiol), and/or the enzymatic pathways that mediate their conversion.

Furthermore, most studies so far focused on the effects of yolk androgens on the offspring, whereas the costs and benefits associated with differential yolk androgen deposition for the mother have received much less attention (Groothuis et al. 2005b; Gil 2008). Our study reveals an association between yolk androgen deposition and female lifespan, indicating that direct consequences for the mother play an important role in the evolution of hormone-mediated maternal effects. The mechanisms underlying the observed association between yolk androgen deposition and female lifespan are currently unknown. If yolk androgen deposition is correlated with the mother's circulating androgen levels, for which there is mixed evidence (Groothuis & Schwabl 2008; Okuliarova et al. 2011), effects of androgens (or their metabolites) on immune functioning (Roberts et al. 2004), metabolism (Buchanan et al. 2001) or the rate of ageing (Kyo et al. 1999) might play a role.

Experimental manipulations of yolk T concentrations in several bird species showed that yolk T can promote post-hatching growth and competitiveness (Groothuis et al. 2005b; Gil 2008; but see Sockman & Schwabl 2000; Pitala et al.

2009). As a consequence, high yolk T transfer has often been interpreted as a form of maternal favouritism (Schwabl *et al.* 1997). The lower recruitment rate of offspring exposed to high yolk T concentrations in our study challenges this view. It suggest that an experimental increase of yolk T in isolation may lead to an overestimation of its beneficial consequences, potentially because the manipulation bypasses trade-offs and constraints associated with exposure to high yolk T (or its deposition) in nature.

High yolk T concentrations are associated with longer natal dispersal distances in another passerine species, the great tit (*Parus major*) (Tschirren *et al.* 2007). If females that deposit higher yolk T concentrations into their eggs have offspring that disperse farther, we might underestimate their LRS. However, we found no indication that differential dispersal might bias our selection estimates: Immigrant females did not deposit higher yolk T concentrations in their eggs than local birds ($F_{1,254} = 0.086$, $P = 0.769$). Furthermore, offspring originating from an egg with high yolk T concentrations were not more likely to move to another forest patch during natal dispersal than offspring originating from an egg with low yolk T concentrations. If anything, exposure to high yolk T concentrations was associated with higher (rather than lower) philopatry ($\chi^2 = 2.739$, $P = 0.098$).

In conclusion, our study is the first to unequivocally demonstrate an association between maternal yolk hormone deposition and fitness in the wild. Thereby, these results provide novel and exciting insights into the adaptive value and evolutionary trajectories of two prominent mediators of prenatal maternal effects in birds and other oviparous species, as well as a mechanistic explanation for the maintenance of variation in maternal investment in natural populations.

AUTHORSHIP

B.T. designed the study; B.T., L.G. & B.D. collected data; L.G. & B.G. coordinated the long-term population monitoring; B.T. and T.G.G.G. performed hormone analyses; B.T. & E.P. analysed the data; B.T. wrote the manuscript; and all other authors commented on the manuscript.

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