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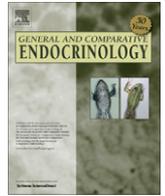
Maternal steroids in egg yolk as a pathway to translate predation risk to offspring: Experiments with great tits

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ABSTRACT

Exposure of mothers to risk of predation can induce phenotypic changes in offspring as shown in several species. We previously found that cross-fostered great tit (*Parus major*) chicks of females exposed to increased predation risk were smaller and lighter, but had faster wing growth than control cross-fostered chicks, possibly improving predator-escaping abilities. Here we examined the possible role of maternal steroids deposited in eggs as an underlying mechanism. We collected eggs from female great tits under either experimentally increased predation risk (PRED) or control treatments (CON) and analyzed the concentration of testosterone, androstenedione, and progesterone in the yolks. PRED eggs contained lower levels of testosterone than CON eggs, but levels of androstenedione and progesterone did not differ. The smaller size and mass of chicks found in the previous study may thus be explained by the lower testosterone concentrations, since yolk testosterone is known to boost growth and development. Alternatively, testosterone may act as a modulator of differential investment into morphological traits, rather than a simple growth enhancer, explaining lower body mass in conjunction with the accelerated wing growth. This could possibly occur concurrently with other hormones such as corticosterone.

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1. Introduction

Maternal effects, the non-genetic influence mothers may exert on offspring phenotype as a response to the prevailing environment [16], may be an important mechanism to prepare offspring for an environment with high predation risk. Examples of predation-induced maternal effects include morphological changes such as the larger "helmets" of *Daphnia cucullata* offspring [1], changes in growth of nestling great tits (*Parus major*) [5], and behavioral changes such as increased antipredator immobility in field crickets (*Gryllus pennsylvanicus*) [28] and tighter shoaling behavior, an anti-predatory defense, in three-spined sticklebacks (*Gasterosteus aculeatus*) [7]. However, the underlying mechanisms by which mothers communicate predation risk to the offspring and change their phenotype are largely still unknown.

In many vertebrate species embryos are exposed to maternal steroids that can modify their development [9]. Since steroid levels vary with the maternal environment they can potentially have a role in predator-induced maternal effects. A few studies suggested that exposure to predation, which raises circulating corticosterone (CORT) levels in great tit females, e.g. [4], may elevate CORT level in

the egg, in turn having an effect on the offspring [25]. However, recently the reliability of egg yolk CORT measurements has been doubted [21]. Additionally, baseline CORT levels may either rise e.g. [26] or drop e.g. [6], due to the presence of predators in the environment, with the bird's perceived danger probably determining the response [4,6,27].

Whereas the low levels of CORT in eggs of small passerines [9], together with the technical problems in measuring egg CORT concentrations [21] make measuring its levels difficult, yolk androgen concentrations are substantial and have been established as an important tool for females to influence offspring phenotype in response to different conditions such as parasite presence, male attractiveness, and breeding density [reviewed by 30]. Furthermore, maternal variation in yolk testosterone (T) and/or yolk androstenedione (A4) concentrations affect growth and behavior in birds by speeding up embryonic development, increasing post-natal growth rate, altering post-hatching sex ratio, boosting nestling begging rates, and sometimes affecting nestling immune responses [8].

Although most research on yolk hormones has focused on effects of T, A4 and progesterone (P4) occurring in even higher concentrations, may have an important role in maternal effects too e.g. [12]. Concentrations of yolk P4, like T, may be lowered as response to maternal stress [2,13]. Here we examined whether the three hormones may be involved in maternal effects induced by predation risk. We increased perceived predation risk for breeding great

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tit females (*P. major*) before and during egg-laying, and measured hormone levels in eggs. We predicted that eggs of females under increased predation risk would have lower androgen concentrations since androgens are often related to increased development rates [8], and since theoretical models on the interaction between growth and predation [22] suggest that growth would be reduced under increased risk of post-fledging predation.

2. Materials and methods

The experiment was performed in a natural population of great tits in a forest near Bern, Switzerland (46°57′N, 7°24′E). The forest was divided into plots distanced from each other by two great tit territories (ca. 120 m) to reduce treatment effects between neighboring plots. We monitored nest boxes closely from the beginning of the breeding season to determine the start of nest building, egg-laying, and incubation.

Six plots were randomly assigned to a treatment of increased perceived predation-risk ('predator'), and six to a control treatment. Perceived predation risk was increased by placing stuffed sparrowhawk models (*Accipiter nisus*; a post-fledging predator of great tits) accompanied by calls played from a portable loudspeaker in eight central locations in each plot. Every day, in the morning or the afternoon (alternated daily), we performed simulations lasting 1.5 h of two sparrowhawks in two of the locations (changed daily). In the control plots we displayed song thrush models (*Turdus philomelos*) and their vocalization. Song thrushes neither predate on great tits, nor compete with them for nesting sites. Both simulated species have been observed occurring naturally in this forest (MC personal observations).

We predefined two thresholds to determine the start of the treatment in a plot: (1) once 5 nests in the plot reached a stage indicating the territory is used and the nest is likely to be finished (newly laid 2 cm layer of fresh moss); or (2) once at least one nest in the plot reached a final stage before laying eggs (egg cup clearly visible, often padded with fur). We closely followed nest boxes by visiting each nest box every third day from the beginning of the breeding season in order to identify the start of nest building and egg-laying, and every day from the 10th day of incubation to determine hatching date. Treatments continued in each plot until hatching of all the nests.

Females of the control and the predator groups were exposed to their respective treatments for a similar number of days before starting to lay eggs (mean ± SE: 9.2 ± 0.7 and 8.7 ± 0.6 days respectively; Wilcoxon Rank-Sum test: W = 1880.5, p = 0.920). The eggs from one nest where egg-laying started before the treatment were excluded from the yolk steroids analysis. On the third incubation day we measured total clutch mass (±0.1 g) and egg number.

For the analysis of yolk hormones, we collected the fourth egg laid in each nest, replacing it with a dummy egg. This is the middle egg in the average clutch of eight, and a good estimate of clutch hormone levels (unpublished data). Collected eggs were placed in −30 °C, and later, for long term keeping, in −80 °C until hormonal analysis. For the hormone analysis we randomly chose 34 and 37 eggs from the control and predator treatments respectively, keeping the rest of the collected eggs for other analyzes.

2.1. Yolk extraction and radioimmunoassay

Yolks were extracted by scraping the egg-shell and albumen from the frozen eggs using a scalpel. Yolk concentrations of T and A4 were quantified by radioimmunoassay (RIA). To extract the hormones, 97–290 mg of yolk/MilliQ mixture (1 + 1) was weighed (±0.001 g). 200 µl of MilliQ water were added, as well as 50 µl of ^{3}H-labeled T to trace the recovery of extracted hormones during the extraction procedure. This solution was incubated for 15 min at 37 °C before being extracted in 2 ml of diëthylether/petroleumbenzin (DEE/PB, 70/30 v/v) by vortexing for 60 s. Extracted samples were centrifuged at 2000 rpm for 3 min (4 °C) to separate the ether phase, the samples were snap-frozen and the ether/hormone phase decanted into a fresh tube. The extraction procedure was repeated twice with an additional 2 ml of DEE/PB, vortexed for 30 s and 15 s, respectively. Next, the extracts were dried under nitrogen. Hormone extracts were rinsed in 2 ml of 70% methanol to precipitate any lipids and stored at least overnight at −20 °C. Subsequently, the tubes were centrifuged, decanted into a fresh tube, re-dried under nitrogen and stored at −20 °C.

Prior to assay, extracts were thawed and dissolved in 400 µl of phosphate-buffered-saline with gelatin (PBSG). From this solution, 50 µl was mixed with scintillation cocktail (Ultima Gold, Perkin Elmer) and radioactivity counted on a liquid scintillation counter. Subsequently, 25 µl of sample (6× dilution) was used for T determination using a kit purchased from Orion Diagnostica ('Spectria 68628', Espoo, Finland). For A4 determination 50 µl of sample (12× dilution) was used, with a kit purchased from Beckman Coulter GmbH ('DSL-3800', Sinsheim, Germany). Standards were prepared using dilution series from pre-prepared stock and ranged from 0.08–20 ng ml^{-1} for T and 0.16–20 ng ml^{-1} for A4.

For P4 determination we used a kit purchased from Orion Diagnostica ('Spectria 06196', Espoo, Finland) with 50 µl of sample (101× dilution). Standards were prepared using dilution series from pre-prepared stock and ranged from 0.47–60 ng/ml.

Recoveries were calculated by comparison to non-extracted ^{3}H-labeled T and averaged 71% (SD = 7%). 'Pools' of yolk were used as external controls and intra-assay CV were 3%, 4%, and 6% for T, A4, and P4 respectively. Parallelism was confirmed for all hormones.

2.2. Statistical methods

For statistical analysis we used general linear models. To account for differences in parental quality and environmental conditions related to seasonality e.g. [20,24,29] we included laying date and its interaction with the treatment, and removed it if not significant. We controlled for yolk-mass when analyzing yolk hormone concentrations, as both have been repeatedly shown to correlate e.g. [10]. Treatment-plot proved non-significant as a random aggregating factor in mixed-effects models, and was therefore excluded. We corrected the non-normal distribution of model residuals by log10 transformation of hormone concentrations.

3. Results

Testosterone concentrations were significantly lower in eggs from the predator treatment, and were not related to laying date or yolk mass (Table 1; Fig. 1). Neither A4 nor P4 concentrations were affected by the predator treatment (Table 1; Fig. 1). Laying date had an almost significant positive effect on A4 concentrations, but a significant positive effect on P4 concentrations (Table 1). None of the interactions in the models were significant (all p > 0.11).

Predator treatment had no significant effect on clutch size and egg mass ($F_{1,43}$ = 0.331, p = 0.568, and $F_{1,109}$ = 1.004, p = 0.319 respectively), with females laying 8.9 ± 1.3 and 8.7 ± 1.6 eggs (mean ± SD) in the control and the predator treatments respectively. Both clutch size and egg mass increased with laying date ($F_{1,43}$ = 5.337, p = 0.026 and $F_{1,109}$ = 1.004, p = 0.009 resp.), but did not intercorrelate (r = −0.055, p = 0.649). The interaction between laying date and the treatment was not significant ($F_{1,109}$ = 0.181, p = 0.672).

Table 1
ANOVA tables and coefficient estimates for measured hormones.

Log10 (pg/mg)	Variable	Estimate (SE)	df	F	p
Testosterone (T)	Intercept	1.892 (0.201)	–	–	–
	Lay date	0.005 (0.004)	1.66	1.295	0.259
	Yolk mass	−0.001 (0.001)	1.66	1.506	0.224
	Predator treatment	−0.076 (0.036)	1.66	4.450	0.039
Androstenedione (A4)	Intercept	1.872 (0.158)	–	–	–
	Lay date	0.006 (0.003)	1.66	3.346	0.072
	Yolk mass	-2×10^{-4} (3×10^{-4})	1.66	0.324	0.571
	Predator treatment	−0.019 (0.029)	1.66	0.448	0.506
Progesterone (P4)	Intercept	3.317 (0.131)	–	–	–
	Lay date	0.006 (0.003)	1.66	5.038	0.028
	Yolk mass	-3×10^{-4} (3×10^{-4})	1.66	0.810	0.372
	Predator treatment	0.022 (0.024)	1.66	0.840	0.363

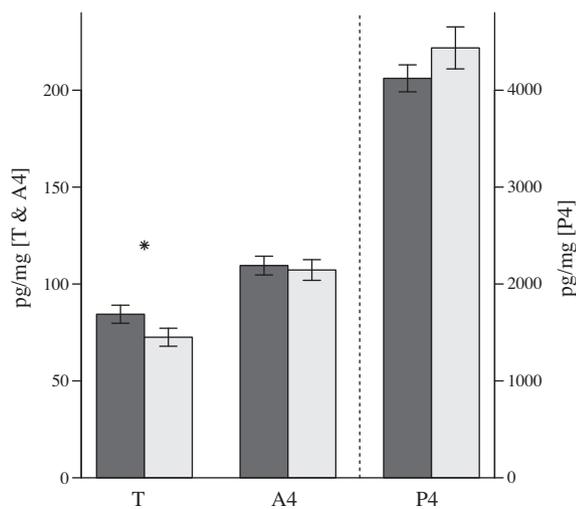Reference level is the control treatment. Lay date refers to 15.3.2009 as day 0. SE = standard error.

Fig. 1. Mean yolk hormone concentrations (±SE) in eggs of mothers perceiving either normal (dark bars) or increased (light bars) predation risk. Asterisk represents significant difference.

Yolk mass did not significantly differ between the treatments ($F_{1,67} = 0.586$, $p = 0.447$), nor was it significantly affected by laying date ($F_{1,67} = 0.168$, $p = 0.684$).

4. Discussion

Here we found lower concentrations of T in eggs of female great tits perceiving experimentally increased predation risk, while A4 or P4 concentrations were not affected. In a previous study on the same nests [5] we found that nestlings from clutches laid under increased perceived predation risk were smaller and lighter than control nestlings, but had faster wing growth resulting in longer wings for first year recruits. Thus the smaller size and mass observed in our previous study suggest negative effects on nestling survival e.g. [17]. Since increasing T levels have often been related to increased growth rate [8], the lower levels found here might explain the smaller size of nestlings of the same clutches. However, we argued that the smaller nestling size and mass combined with the longer wings could be seen as an adaptive response, as they could have increased survival in a predator-rich environment through reduced wing load and improved maneuverability [3]. Thus T levels, under this interpretation, may underlie an adaptive maternal effect. Since nestling wing growth was accelerated in the broods with eggs analyzed here [5], this would alter the covariance among nestling morphological traits. Similar changes in covariance among traits due to experimentally increased T levels have been found among male secondary sexual traits in pheasants (*Phasianus colchicus*) [23]. Thus, T may modulate phenotype development leading to differential investment in morphological traits, rather than simply augmenting development quantitatively. If CORT indeed rises in eggs of females exposed to predation risk [25], the two hormones may work concurrently in determining offspring phenotype yet this has still to be established.

Lower yolk T levels may also adaptively adjust behavior to predation risk. Behaviors such as chick begging and competitive behaviors are affected by yolk T levels [reviewed in 8]. Since increased competitive behavior and begging, which is accompanied by loud calls, may increase risk of predation e.g. [15], reduction of these offspring behaviors by reducing yolk T levels may be adaptive in a predator-rich environment.

How birds regulate yolk steroid levels is unknown [see 11], but there is recent evidence that androgen concentrations in the egg are independent from those in the maternal circulation [see also review by 11,19]. In mammals, ovaries contain glucocorticoid receptors, and their activation (in our case by elevation of corticosterone due to exposure to predators) may down-regulate T production in the follicle wall of the ovum. Consistent with this is the finding that elevation of corticosterone in the maternal circulation during egg production in the chicken suppressed yolk T and P4 deposition in the eggs [13].

We found no effect of our treatment on A4 concentrations. Since levels of A4 are much higher than those of T, with the former having a very much lower affinity to the androgen receptor, modulation of the conversion of A4 to T is more relevant than modulation of A4 levels itself. It has indeed been suggested that yolk A4 may primarily act as a source for biological active steroids such as T, 5-alpha-dihydrotestosterone and estradiol [10].

P4 levels were even higher than A4 levels as is the case in other species, its levels increased with the season, but there was no effect of treatment. Although being biologically active in adult birds [18], with some evidence that it stimulates early embryonic development in mammals [14], its role in avian embryonic development is largely unknown.

Thus, our results suggest that female birds may use T as a tool to adapt offspring phenotype to predation risk. Yolk T may act not as a quantitative booster of growth, but rather as a modulator of differential investment in morphological traits, possibly alongside CORT. However, data to support the latter is currently lacking.

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