Selection on personality in a songbird affects maternal hormone levels tuned to its effect on timing of reproduction
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Supplemental material concerning full methods, additional results and further discussion of the paper “Selection on personality in a songbird affects maternal hormone levels tuned to its effect on timing of reproduction” by Groothuis et al.

MATERIAL AND METHODS

Subjects and breeding

The great tit is a territorial, non-migratory passerine bird (body mass: 16-20 g) inhabiting woodlands and parks (Cramp et al. 1993). The sampled birds belonged to the 3rd, 4th and 5th generation of a program of artificial selection from a wild population that started in 1993 (Drent et al. 2003). Birds were selected for a Bold versus Shy behavioural phenotype (also named Fast and Slow explorers in other papers) and a line difference in the selection criterion was confirmed in adulthood (Carere et al. 2005). Alternating pairs of the two lines were housed in adjacent identical outdoor aviaries from the end of winter (February) onwards and experienced the same environment. For details of housing and feeding see Carere et al. (2005).

Egg collection and breeding parameters

We collected 66 eggs of first clutches from 6 pairs of the Shy and 9 pairs of the Bold line in two subsequent years kept in identical conditions in adjacent aviaries hosting alternatively Shy and Bold pairs. The majority of females were adult (more than one year old) experienced breeders. Great tits lay one egg per day and we inspected nest boxes daily and collected fresh eggs, immediately replacing them with dummies. We froze marked eggs at -20°C within a few minutes from collection. For each pair we recorded: 1) onset of nest building (date when first nesting material was found in the nestbox); 2) laying date (date of first egg laid); 3) onset of incubation (date when female was on the nest cup in
incubation posture or when eggs were warm); and 4) clutch size. Finally, 5) we estimated
the expected degree of hatching asynchrony as follows. Every day we checked the nest box
for egg laying and whether the bird was incubating (female on the nest, or eggs warm). We
used days of incubation before clutch completion as the expected degree of hatching
asynchrony.

Yolk steroid analyses

Yolks were separated from albumen, weighed to the nearest 0.01 g and then homogenised
with an equal volume of distilled water. The procedures for extraction and
radioimmunoassays of testosterone (T), androstenedione (A₄), 5α-dihydrotestosterone
(DHT), corticosterone (B) and 17β-estradiol (E₂) in a sub-sample of the yolk-water
homogenate were as previously described (Schwabl 1993). Eggs were analysed in two
batches with eggs of both selection lines balanced over both batches. Intra-assay
coefficients of variation were for A₄: 7.5; DHT: 9.0; T: 8.5; E₂: 10.3 and B: 12.1. Mean
recoveries were 67.5, 45.3, 68.3, 71.1, and 48.3 %, respectively.

Selection criteria: exploration score

A full description of the exploration tests used for selection are provided elsewhere
(Drent et al. 2003; Carere et al. 2005). We used the sum of three criteria, briefly
described as follows: 1) The time needed to visit four of five trees in an unfamiliar
room; 2) A composed measure of latency and distance of approach to a penlight battery
and 3) a 8 cm pink rubber toy in the home cage. Realized heritability in the lines was
0.54. From the 3rd generation onward lines differed on average by more than 10 units of
the exploration score (Drent et al. 2003).
Data analysis

Hormone concentrations were log 10 - transformed to obtain normal distributions. We used hierarchical linear models (Bryk & Raudenbush 1993). These models accommodate unbalanced data allowing analyses of variances and covariances, while simultaneously taking the nested relationships of offspring within clutches of individual females into account. To accommodate the hierarchical nested structure of our data set individual eggs were nested within clutches, and clutches within females (while two females were sampled in two different years). The MLwiN software accommodates unbalanced data, and we were unable to collect eggs of each position in the laying sequence from each female. We performed a backward stepwise procedure, retaining only those predictors in the model that were significant (p<0.05). The significance of predictors was tested using the difference in deviance (or –2log-likelihood values) between two models, the one that included and the one that did not include the predictor tested. The difference in deviance is distributed as Chi-square, with the difference in number of predictors between the models as degrees of freedom. We tested associations between concentrations of different hormones by Pearson correlation coefficient. We analysed line differences in breeding parameters (some of which could not be obtained from all pairs) with one-way ANOVA. We detected no effect of age or year or their interaction with line or egg sequence in any of the data sets.

RESULTS

E₂ concentrations (7.7 ± 0.44 pg/mg yolk) were lower than those of androgens (A₄: 37.38 ± 2.89; T: 16.67 ± 1.22; DHT: 21.64 ± 1.47), while B concentrations (0.64 ±
0.05) were very low. The levels of some of these hormones were positively correlated with each other, in particular T and its precursor A_4 (Table 1). Their analysis in relation to selection line yielded similar results. Therefore, and because T has a higher affinity to the androgen receptor than A_4 (Sonnefeld et al. 2006), we have reported in the paper only data on T.

**DISCUSSION**

These results are obtained in birds from the same selection experiment. No replicas of these lines have been made and the results should need validation from other experiments (see p. 318, Falconer & Mackay 1996). However, information from two other sources indicate that the results are not an accidental by-product of the selection, due to genetic drift and the characteristics of the birds from which the selection originated. i) data from unselected birds, both juveniles and adults collected from the field (e.g. Marchetti & Drent 2000; Carere & van Oers, 2004). ii): data from another selection experiment on risk-taking behaviour in the same species that yielded similar personality profiles (van Oers et al. 2004; review in Groothuis & Carere, 2005).

As regards the other steroids, E_2 occurred in much lower levels than the androgens, perhaps because it would interfere with sexual differentiation, which in birds is sensitive to estrogens (Carere & Balthazart 2007). Levels of B were extremely low, perhaps because early exposure to glucocorticoids can have detrimental effects on the nestlings (Love et al. 2006). The low levels of B may shed some light on the process of hormone accumulation in the egg. Of the 5 steroids, B is produced in the adrenals only and not in the follicle wall of the ovaries in which the eggs are yolked. Therefore, and in contrast
to the androgens and the estrogen, which are synthesized in the ovarian follicle, B has to be transported via the circulation to the proliferating ovarian eggs. In female blood plasma, concentrations of B are normally much higher than those of androgens and E₂. Therefore, the extremely low levels of B in the yolk suggest that passive transfer of steroids from the maternal circulation into the oocyte only marginally contributes to concentrations of steroids in the egg. We suggest that avian mothers have evolved a more direct mechanism by which eggs can be provisioned with gonadal steroids and as shown here, this can be selected for.

References


Table 1. Pearson correlation coefficients for yolk steroids in great tit eggs (n = 66). Significant p-values are shown in bold type. * p < 0.05; ** p < 0.01

<table>
<thead>
<tr>
<th></th>
<th>DHT</th>
<th>A4</th>
<th>T</th>
<th>E2</th>
<th>B</th>
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<td></td>
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<tr>
<td>T</td>
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<td>0.24</td>
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<td>0.29*</td>
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<tr>
<td>B</td>
<td>0.09</td>
<td>0.31*</td>
<td>0.21</td>
<td></td>
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N = 66 eggs