Divergent selection for inherent fearfulness leads to divergent yolk steroid levels in quail

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Summary

Avian yolk steroid levels may vary according to maternal condition or environmental factors,
causing epigenetic modulation of offspring phenotype. Here we test whether artificial selec-
tion based on divergent duration of tonic immobility (TI) in Japanese quail (i.e., divergent
inherent fearfulness) is correlated with divergent levels of yolk steroids. We analysed yolk
steroid levels and egg quality in quail selected for either long or short duration of TI. Yolk
steroids, i.e., immunoreactive progesterone and androstenedione, were found to be signifi-
cantly less concentrated in eggs of the high inherent fearfulness line compared to eggs of the
low inherent fearfulness line. A similar trend was found with testosterone levels. Larger eggs
with lighter eggshell were also found in more fearful quail. Hence, the selection for diver-
gent fearfulness has led to correlated changes in yolk steroid levels and egg quality. These
data suggest that hormones of maternal origin, egg quality and genetic background may all
contribute to line differences in phenotype. A modulation of progesterone concentrations by
selection for behaviour in egg yolk is reported here for the first time. Although the effect of
this hormone on avian embryos remains unknown, we argue it may have significant effects
on phenotypic outcome.

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Introduction

Prenatal exposure to sex steroids of maternal origin can affect individual behavioural, physiological or morphological characteristics and subtle differences in the hormonal environments of embryos may cause substantial phenotypic variability (Groothuis & Carere, 2005; Groothuis et al., 2005; Kaiser & Sachser, 2005). A number of factors are known to modulate the levels of yolk steroids in avian species, including breeding density (e.g., Schwabl, 1997; Groothuis & Schwabl, 2002), frequency of social intrusions (Mazuc et al., 2003), maternal social status (Müller et al., 2002), diet (Verboven et al., 2003) and male attractiveness (Gil et al., 1999). In addition, maternal age (Pilz et al., 2003) and parasite load (Tschirren et al., 2004) may modulate yolk hormone levels and offspring phenotype. In altricial species of different orders, correlational, as well as experimental studies have found that prenatal exposure to androgens can influence growth, activity levels, begging behaviour, the frequency of aggressive and sexual displays (e.g., Schwabl, 1993, 1996; Eising et al., 2001, 2006; Eising & Groothuis, 2003; Pilz & Smith, 2004; Rubolini et al., 2006) social dominance among nest-mates (Schwabl, 1996) and modulate neophobic responses (Tobler & Sandell, 2007).

Precocial species have received far less attention. Recent studies have highlighted a prenatal influence of androgens both on social and emotional behaviour and on learning tasks. Daisley et al. (2005), for example, reported that young Japanese quail (*Coturnix japonica*) hatched from eggs injected with testosterone, independent of sex, have a lower level of emotional reactivity (with shorter durations of TI), are less dependent socially and perform a detour task faster, indicating a shift towards 'proactive' or 'bold' phenotypes. Similarly, lower fear responses to an open field challenge in quail from testosterone treated eggs have been reported by Okuliarová et al. (2006). This effect of androgens appeared, however, to be age-dependent, as increased fearfulness with higher TI duration was reported in 2-day-old quail from testosterone treated eggs (Okuliarová et al., 2007). Increasing progesterone levels in eggs has also been found to alter embryonic growth in chicks (Ahmad & Zamenhof, 1979; Renden & Benoff, 1979). Progesterone is a precursor of androgens, and is secreted in the granulosa cells of the pre-ovary follicles (Huang & Nalbandov, 1979). Although it is the principal hormone surrounding avian embryos in the early stage of development (Lipar et al., 1999; Lipar, 2001; Möstl et al., 2001) it has received very little attention in the literature.

Little is currently known about the potential genetic basis of variation in yolk steroid levels, although recent reports indicate such influence. Indeed, Japanese quail selected for high plasma corticosterone response have been found to deposit high levels of corticosterone in their eggs (Hayward et al., 2005). Quail selected for high social reinstatement (SR) behaviour laid eggs with higher levels of yolk testosterone (Gil & Faure, 2007). Another interesting model for investigation of the relationship between hormones and behaviour is provided by lines of Japanese quail divergently selected on the basis of the duration of tonic immobility, an index of fearfulness. We studied whether this selection on behaviour has also affected yolk hormone concentrations, egg mass and eggshell.

Selection was performed between 9 and 10 days of age by measuring the duration of tonic immobility. One line was selected for long durations of TI (long tonic immobility (LTI) line) and the other line for short durations of TI (short tonic immobility (STI) line): a full description is given by Mills & Faure (1991). The duration of the tonic immobility reaction is considered to be a standard and robust measure of fearfulness (Gallup, 1977; Jones, 1986, 1987). The selection program modified the general underlying fearfulness rather than exerting only specific effects on TI (Jones et al., 1991). Chicks and adults of the LTI line show more behavioural inhibition in the open-field, longer emergence times on tests of emergence (Jones et al., 1991; Richard-Yris et al., 2005), longer latencies to approach novel food, are more disturbed by the sudden introduction of a frightening stimulus into their home cages (Launay, 1993) and express less exploratory behaviour (Richard-Yris et al., 2005). The LTI quail, thus, have a higher emotional reactivity (used in its restrictive sense i.e., propensity to express fear responses, Bouissou et al., 1994; Jones, 1996) compared to STI quail. The behavioural profiles obtained could be associated with the extremes of an axis of behavioural types found in both captive and wild populations of other species: reactive–proactive, slow–fast and passive–active coping styles (see Koolhaas et al., 1999 for a review). Reactive birds are, for example, more cautious in face of novelty and express less exploratory behaviour (Great tit, *Parus major*, Dingemanse & de Goede, 2004) and, thus, closely resembled birds of the LTI line.

Variation in fearfulness within populations of animals has an adaptive-ecological value since it can allow species to cope with changing environments (Gallup & Suarez, 1980). Quail selected for high or low level of fearfulness adjust their reactivity level to different environmental conditions

(e.g., enrichment) indicating that genetic differences do not cancel the advantage of facultative adjustment. Indeed, variations in fear behaviour can be engendered by non-genomic maternal effects (Daisley et al., 2005; Okuliarová et al., 2006) via yolk hormones and can be accompanied by variations in general egg quality, like egg size or yolk weight (Minvielle et al., 2002). In quail and other domestic species variations in fear behaviour are also genetically determined (e.g., Gallup, 1974; Gallup & Suarez, 1980) and we hypothesize that variations in yolk hormone levels, mediating maternal effects, could also be the result of genetic inheritance. We examined the potential relationship between selection, yolk hormone levels and the general quality of eggs with the expectation of finding divergence between STI and LTI quail lines and more androgen in less fearful birds.

Materials and methods

Animals and housing

We obtained eggs from females (7 months old) of the 43rd generation of the STI and LTI lines of Japanese quail, selected and maintained at the French National Institute of Agricultural Research (INRA) of Tours (France, see Mills & Faure, 1991 for a full description). The initial stock for the original breeding program was formed from two commercial strains that were reciprocally outbred. From these birds, one part was subjected to divergent selection for duration of TI weighted for independence from social reinstatement (SR) behaviour, and another part to divergent selection for SR behaviour weighted for independence from TI responses (SR behaviour is assessed by the distance run in a treadmill test at 6–8 days of age. The selection for TI duration is performed with the precaution to maintain the same level of SR behaviour between the two divergent lines). These animals were used as the parents of a long TI duration (LTI) line, a short TI duration (STI) line, a high SR (HSR) line and a low (LSR) line. Animals of next generations of the LTI and STI lines were submitted to directional selection for TI duration, suggesting that these factors can be separated at the genetic level and selected for independently (Mills & Faure, 1991). During the tonic immobility test, animals (9–10 days of age) are restrained on their backs for 10 s and then released. When more than 10 s lapsed between release and escape, duration of TI is recorded; if not, induction is attempted again. A score of zero seconds is

given when no TI is attained after five attempts. When a bird does not stand up within 5 min, a maximum duration of 300 s is scored. This manipulation induces a reversible catatonic state, the duration of which is positively correlated with general underlying fearfulness (Jones et al., 1991; Mills et al., 1994). Each generation of the divergent lines consisted of 20 males and 40 females. At generation 42, mean $\pm$ SD TI durations were 241 ± 82 s for the LTI line and 12 ± 18 s for the STI line, clearly showing no overlap between lines.

Birds were housed in standard cages ($24 \times 21.5 \times 17$ cm) in groups consisting of two females and one male. Food (commercial game bird diet formulated as recommended by INRA, 1984) and water were provided ad libitum with a light/dark cycle of $16:8$ h. The temperature was maintained at $20 \pm 2°C$.

Egg sampling and analysis

Domestic Japanese quail lay eggs on a daily basis (absence of clutch) and twenty eggs per line were collected randomly from the breeding population the same day, with only one egg per female being sampled. Eggs were frozen at $-20°C$ from collection until steroid extraction. The mass of each egg was measured on electronic scales to the nearest 0.001 g. As described by Lipar et al. (1999) and Hackl et al. (2003), the frozen yolk was separated from the albumin and weighed. Eggshells were also separated, washed with water to withdraw the albumin, dried and weighed. The weight of the albumin was determined by subtracting the weight of the eggshell and the yolk from the total mass of the egg. To measure the concentrations of progesterone and androgens in the yolk we used methods similar to that described by Möstl et al. (2001) and Hackl et al. (2003). As the distribution of hormones varies between yolk layers (Lipar et al., 1999; Möstl et al., 2001; Hackl et al., 2003), the yolk was cut in half and an entire homogenised half yolk was assayed to obtain a representative sample of yolk. To extract steroids, after thawing, each yolk sample was suspended in 10 ml of water and vortexed twice for 30 s. Samples were then stored overnight at 4°C. Afterwards, samples were vortexed and 1 ml of the suspension was transferred to a new vial. The suspension was then diluted with 4 ml methanol, vortexed for 30 min and stored at $-20°C$ overnight to precipitate apolar lipids. After centrifugation ($-15°C$, $2500 \times g$, 10 min) 10 μl of the supernatant were used for enzyme immunoassays (for full descriptions of antibodies and validation see Palme & Möstl,

1993; Hirschenhauser et al., 1999; Möstl et al., 2001). We measured yolk testosterone concentration in five assays, and androstenedione and progesterone in six assays. The inter-assay coefficients of variation were 19.5%, 10.4% and 15.9%, respectively, for the low level pool and 12.3%, 5.5% and 15.3% for the high level pool. The intra-assay variation was 8.5%, 4.2% and 9.2%, respectively.

Data analyses

Data were analysed using Statview 5.0 (SAS Institute). As normality was not achieved with our data, nonparametric statistics were applied. Mann–Whitney U-tests were used to compare the data from STI and LTI quail and Spearman correlations evaluated a potential correlation between the levels of the different steroids assayed.

Results

Significantly lower concentrations of progesterone (Figure 1a) and androstenedione (Figure 1b) were found in the LTI line compared to the STI line. A similar trend (Mann–Whitney U-test, $z = -1.86$, $p = 0.06$) was observed for testosterone (Figure 1c). In both lines all of these hormones had a significant positive correlation with one another (Spearman, $p < 0.05$ for all tests). If we analysed hormone content instead of hormone concentration, a similar pattern was observed for progesterone and testosterone (progesterone: 4773.73 ± 412.87 ng/yolk for LTI line and 6416.25 ± 501.4 ng/yolk for STI line, Mann–Whitney U-test, $z = -2.75$, $p = 0.005$; testosterone: 41.18 ± 3.82 ng/yolk for LTI line and 58.41 ± 6.92 ng/yolk for STI line, Mann–Whitney U-test, $z = -1.65$, $p = 0.005$). Similar levels were found for androstenedione (563.21 ± 36.44 ng/yolk for LTI line and 680.69 ± 50.29 ng/yolk for STI line, Mann–Whitney U-test, $z = -1.62$, $p = 0.1$).

Eggs from LTI females were significantly heavier (11.50 ± 0.29 g and 10.74 ± 0.2 g, respectively, Mann–Whitney U-test, $z = -2.03$, $p = 0.037$) and tended to contain both more yolk (3.42 ± 0.13 g and 3.1 ± 0.07 g, respectively, Mann–Whitney U-test, $z = -1.84$, $p = 0.06$) and albumin (7.15 ± 0.19 g and 6.64 ± 0.12 g, respectively, Mann–Whitney U-test, $z = -1.89$, $p = 0.058$) than eggs of the STI females. LTI females, on the other hand, laid eggs with significantly lighter eggshells (0.93 ± 0.02 g and 0.99 ± 0.01 g, respectively, Mann–Whitney U-test, $z = -2.35$, $p = 0.01$).
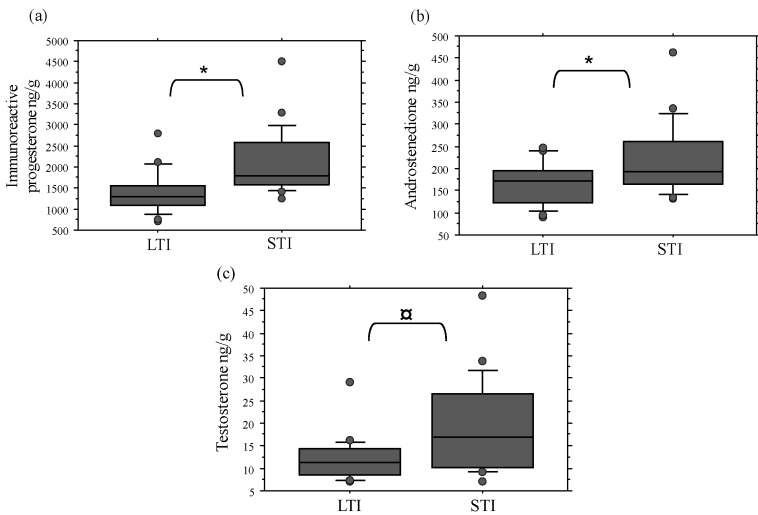
Figure 1. Boxplots of the immunoreactive progesterone (a), androstenedione (b), and testosterone (c) concentrations (ng/g) in eggs of the LTI and STI lines. ∗ Mann–Whitney, $p < 0.05$. ¤ Mann–Whitney, $0.05 < p < 0.1$.

Discussion

We found clear differences in yolk hormone concentrations and content between the eggs laid by quail selected between nine and ten days of age for a short or long duration of tonic immobility. For the first time, variations in yolk progesterone levels following selection for behavioural phenotype were found.

Birds with higher emotional reactivity laid eggs with lower yolk steroid concentrations. This is of particular interest because emotional reactivity is a key component of coping style (i.e., a coherent set of behavioural and physiological stress responses which are consistent across time and/or context, Koolhaas, 1999). In field studies, higher yolk androgen levels are usually found following female exposure to social stressors (Schwabl, 1997; Wittingham & Schwabl, 2002). Our birds, however, were not exposed to any highly stressful situations. Furthermore, as LTI birds are more sensitive to stressful situations, more androgens would have been expected in eggs of this line. Moreover, both lines were housed under identical conditions and no differences in social behaviour have been found between these lines (Mills & Faure, 1991). Recently, genetic differences linked with the duration of tonic immobility were found between the STI and LTI quail (Beaumont et al.,

2005). The differences in behaviour between lines are probably shaped by the combined action of genes and prenatal exposure to maternal hormones. Our study, as well as other recent reports with birds selected on the basis of their behavioural profiles (Gil & Faure, 2007; Groothuis et al., 2008) strongly suggests a genetic component to variations in yolk hormone levels. Genes coding for fearfulness may code directly for hormone production. We cannot completely rule out the possibility, however, that selection has engendered differences in sensitivity to the environment (Jones et al., 1991; Richard-Yris et al., 2005) that in turn affect hormone production. Brief human disturbances, for example, have been found to affect yolk hormone levels in unselected quail (Bertin et al., 2008).

Our results confirm results obtained in quail selected on the basis of social motivation, in which testosterone was found to be more highly concentrated in eggs of females selected for a high level of social motivation (Gil & Faure, 2007). These data support the hypothesis that variations in yolk androgen levels correlate with selection for behavioural traits. At present, environmental factors and maternal condition are the main factors that have been investigated and identified as sources of variation in yolk steroid levels in avian species. Furthermore, variations in androgen levels have mostly been investigated in altricial species with asynchronous hatching. As yolk androgens have been found to increase competitive capabilities of chicks in many species, a dominant hypothesis about the function of hormone variations concerns compensation for the disadvantages of late hatching birds that have to compete with sibling in the nest for food (Hubert & Schwabl, 2003). Our data with precocial quail suggest that yolk androgens may also vary according to maternal genotype and in the absence of hatching asynchrony. The behavioural profiles of STI quail closely resemble those of other bold or proactive profiles obtained after androgen injection (Daisley et al., 2005; Groothuis et al., 2005) and, as expected, more androgens were found in STI eggs. The combined action of genetic and non-genetic factors, such as egg nutrients and hormones of maternal origin, thus, likely contributes to the behavioural divergence between lines.

We found evidence of a more pronounced effect on the precursors of testosterone (progesterone and androstenedione) than testosterone itself in eggs of birds selected for inherent fearfulness. This result contrasts with the findings of Gil & Faure (2007), where eggs of quail selected on the basis

of their social motivation were found to differ in yolk testosterone concentrations and not androstenedione concentrations (progesterone levels, yolk, albumen and shell masses were not measured). Selection for social reinstatement behaviour and selection for tonic immobility have probably affected steroid synthesis in different ways. As in many species, we found evidence of higher levels of androstenedione than testosterone in quail. Androstenedione may be converted to testosterone by the embryo itself and, thus, may serve as a source of biologically potent androgens (Groothuis & Schwabl, 2002). The affinity of this hormone for androgen receptors is lower than testosterone (Sonneveld et al., 2005) and its action on chick phenotype remains largely unknown. Some data have, however, suggested a negative correlation with nestling growth (American kestrels, Sockman & Schwabl, 2000) and some authors suggest an effect on the competitive abilities of chicks (*Guira cukoo*, Cariello et al., 2006). Hatchlings from the STI line were found to be smaller than hatchlings of the LTI line (unpublished data and personal observations) and, although further investigation is needed, the higher androstenedione concentrations in STI eggs may contribute to this difference. However, this difference cannot be linked solely to yolk hormones, as differences in egg size have been observed between lines. It is also interesting to point out that whereas testosterone levels reported in quail selected for TI or SR behaviour overlap and are within the ranges observed in unselected quail (between 4 and 30 ng/g; Hackl et al., 2003; Hayward et al., 2005), androstenedione levels differ between selected and unselected quail lines. Eggs of quail selected for divergent TI durations have higher levels than unselected quail: 582.20 nmol/kg for the LTI line and 772.87 nmol/kg for the STI line compared to 453 nmol/kg for unselected quail (Hackl et al., 2003). Conversely, the levels observed in quail selected for divergent SR behaviour (around 111 nmol/kg, Gil & Faure, 2007) are below the ones observed in unselected quail. Although we cannot determine whether hormones determined behaviour or whether differences in behaviour determined yolk hormone levels, selection for two different behavioural traits in birds from the same original population has led to divergent yolk androstenedione levels.

We also found evidence, for the first time, of a difference in yolk progesterone following selection for behaviour. This hormone is secreted in the granulosa cells of the pre-ovary follicles (Huang & Nalbandov, 1979). The germinal disc and overlying granulosa cells are associated closely in the follicle (Tischkau & Bahr, 1996) and the germinal disc is located in the outer

layer of the yolk where the highest progesterone concentrations and lowest concentrations of androgens were measured (Lipar et al., 1999; Möstl et al., 2001). Progesterone can be converted into androgens by side-chain cleavage. One possible explanation for the higher level of progesterone observed in the STI line could be a higher production of the follicular wall. It is also interesting to point out that the levels of progesterone observed in our selected quail lines were well above values observed in unselected quail: 15.18 nmol/yolk for LTI line, 20.4 nmol/yolk for STI line compared to 4 nmol/yolk for unselected quail (Hackl et al., 2003). In mammals and birds, progesterone is known to influence embryonic growth (Ahmad & Zamenhof, 1979; Renden & Benoff, 1979; Ghosh et al., 1997; Lonergan et al., 2007) and is considered to be an anxiolytic steroid (Reddy et al., 2005). Due to the similarities of steroid molecules across species and the large quantity observed in yolk, particularly around the germinal disc, we argue that its potential influence on avian behavioural phenotypes is large. Testosterone remains the target of most investigations, but injecting eggs with progesterone would help to determine the influence of its precursors on the development of offspring behaviour.

The selection based on fearfulness traits had consequences for the general quality of eggs, with larger eggs laid by more fearful birds. This pattern of variation in egg size and egg components confirms previous reports (Minvielle et al., 2002). Carotenoids (Biard et al., 2007), for example, have been the subject of previous investigations, but albumin, eggshell quality and egg size have rarely been considered. Egg size and/or albumin can, however, significantly affect offspring hatching and growth (Dzialowski & Sotherland, 2004; Ferrari et al., 2006), as can yolk testosterone (Eising et al., 2001). Egg size and egg components may, thus, be important to take into consideration when assessing hormone mediated maternal effects. Consistent differences in coping styles have been reported in many wild and captive species (Dingemanse et al., 2004) and are thought to be subject to natural selection (Koolhaas, 1999). Artificial selection may to a certain extent mimic processes of natural selection and whole egg quality may, thus, vary in association with variations in behavioural phenotypes in avian species. Although cause and effects could not be disentangled, our data suggest a combination of genetic and epigenetic influences on the behavioural divergence observed between selected lines of Japanese quail.

Acknowledgements

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