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Effect of Increasing Yolk Testosterone Levels on Early Behaviour in Japanese Quail Hatchlings

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Abstract

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The aim of our study was to investigate effects of increased testosterone content in egg yolk on early behaviour of 1- and 2-day-old Japanese quail. Three different doses of testosterone (0.25; 2.5 and 25 ng), not exceeding a physiological range, were examined in three separate experiments. Testosterone propionate dissolved in 20 µl olive oil was injected into the yolk before the onset of incubation.

Behaviour of newly hatched chicks was recorded in response to both a novel environment in the open-field test and manual restraining in the test of tonic immobility (TI). Behavioural consequences of embryonic exposure to elevated testosterone were observed in the open-field test in all three experiments which indicated inhibition of behavioural responses in hatchlings. Birds treated with testosterone *in ovo* displayed longer latency to leave the start square, decreased locomotor activity, enhanced defecation and lower number of distress calls as compared to control birds. In TI test, the influence of treatment was manifested at the highest concentration only. Hatchlings from testosterone treated eggs expressed longer duration of TI and required less attempts to induce TI in comparison with the control group.

Our results demonstrated increased fearfulness of Japanese quail chicks hatched from eggs with experimentally elevated testosterone content. The effect is specific for a short period after hatching since previous studies reported stimulatory effect of yolk testosterone on behaviour of Japanese quail later in ontogeny.

Maternal hormones, testosterone, quail, behaviour, ontogeny

Early postembryonic period is a critical stage in development of avian species mainly because it is carrying much more risk than protected conditions in the egg and newly hatched chicks must cope with unfamiliar challenges from the external environment. Appropriate behavioural responses of individuals may reflect epigenetic adaptations that are formed as a result of conditions to which they are exposed in embryonic milieu (Horton 2005). Therefore laying hens may influence phenotypic development of their progeny not only by providing basic genetic information but also by depositing nutrients and biologically active substances into their eggs. It is well known that lipid soluble vitamins and antioxidants are necessary for successful embryonic development, including posthatching growth, health status and future performance of hatchlings (Surai et al. 1998; Karadas et al. 2005). Current research is focused on maternal hormones as other significant components of egg yolk that may substantially affect an ability of offspring to cope with their environment (Groothuis et al. 2005). Steroid and thyroid hormones are predominantly important in this consideration since their mechanism of action involves a control of gene expression. Moreover, early exposure to these hormones usually produces long-lasting consequences later in ontogeny (Schlinger 1998). Experimentally enhanced androgen concentrations in eggs have been shown to stimulate postincubation growth and begging

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behaviour in hatchlings of altricial and semiprecocial wild-living birds (Schwabl 1996; Eising and Groothuis 2003).

Behavioural data from precocial species indicate that young quail chicks hatched from testosterone treated eggs exhibit less fearfulness in both a novel object test and tonic immobility test. In other words, they adopt "proactive" behavioural strategy (Daisley et al. 2005). The conclusion is supported also by our previous study in male Japanese quail. Nine-day-old birds hatched from eggs treated with testosterone showed increased activity in response to a novel environment (Okuliarová et al. 2006). Thus, maternal hormones may participate in forming individual responses to the same stimulus that means they may increase variability in phenotypic traits, which in turn increase survival and reproductive success (Groothuis and Carere 2005).

Most studies in this field usually include treatment of eggs with only androgen concentration nearing the upper physiological limit. In the present study, we examined three different doses of testosterone, with one hundred-fold difference between the lowest and the highest concentration, that still were in a physiological range. The specific effects of increasing hormone content in eggs were assessed using this experimental approach. Based on the results of previous research, it was hypothesized that experimentally enhanced yolk testosterone content will have a stimulatory effect on early behaviour in 1- and 2-day-old quail hatchlings.

Materials and Methods

Our study consisted of three separate experiments (E1, E2 and E3) using Japanese quail (*Coturnix japonica*) as a model of poultry species. Identical experimental protocols were utilized for all three experiments, differing only in the dose of testosterone administered into the egg yolk.

Quail eggs were obtained from a breeding colony housed at the Institute of Animal Biochemistry and Genetics, SASci, Slovakia. Per each experiment, quail were randomly assigned into one of two groups, a testosterone-treated (T) and a control (C), respectively. Just prior to incubation, all eggs of the testosterone-treated group were injected with the required concentration of testosterone propionate (Agovirin, Léčiva, Czech Republic) dissolved in 20 µl olive oil. In E1, eggs of the T-group (n = 107) received 0.25 ng testosterone and eggs of C-group (n = 72) were left intact. In E2, the dose of 2.5 ng testosterone was administered into each egg of T-group (n = 35) and eggs of C-group (n = 40) were injected with 20 µl olive oil. In E3, eggs of T-group (n = 93) were treated with 25 ng testosterone and eggs of C-group (n = 90) received 20 µl olive oil.

At first, the eggshell was disinfected with 70% ethanol and injections were made through the blunt pole of the egg into the yolk. After injection, the hole was sealed with paraffin. Eggs were placed into a forced draught incubator (Bios Midi, Czech Republic) with automatic turning and incubated in constant darkness at 37.0 ± 0.2 °C and relative humidity of 50-60%. After hatch, quail chicks were leg-banded with colour rings and placed in separate cages according to their treatment group. Birds were housed under continuous light and temperature maintained at 35-37 °C. Quail were fed young turkey mash and water was provided *ad libitum*. Chick body weights were recorded before starting the behavioural tests on day of hatch.

Open-field test

The open-field test was performed among a sampling of 1-day-old hatchlings outside the breeding room. Quail (62) were placed in a wooden box measuring 25 × 25 × 25 cm (w × l × h) with white painted walls and floor divided into 25 equal squares. The box was situated in an area with temperature corresponding to the temperature in the breeding room. Each bird was individually placed into the central square of the box and after 10 seconds its behaviour was recorded by video camera for 15 minutes. The following behavioural categories were analysed from the videotapes: locomotor activity (number of lines crossed), vocalization (number of distress calls emitted), pecking (number of both wall- and floor-pecks), attempts to escape (number of jumps), defecation (number of defecations) and latency to explore (leave the start square), latency to vocalize (first call) and latency to defecation.

Tonic immobility test

Quail hatchlings (168) were tested for their tonic immobility (TI) responses between 1 and 2 days of age. Each bird was removed from its cage mates and carried individually into a separate testing room. The state of TI was induced by placing the bird on its back in a cardboard V-shaped model with one hand over its body and another one slightly over its head (Jones et al. 1994). The hand pressure was released after 10 s and the duration of TI was measured when chick stayed immobile longer than 5 s. Maximum score for TI duration was 300 s. If TI could not be induced by three repeated attempts, the bird was returned back into the home cage and its TI duration was given 0 s. The number of attempts required to induce TI as well as duration of TI was recorded.

Statistical analysis

All data were examined for normality and homogeneity of variance and analysed using either Student's *t*-test or non-parametric Mann-Whitney test.

Results

Experimental injections had no impact on hatch success of chicks between controls (40.3%, 57.5% and 50.0%) and testosterone-treated (32.7%, 54.3% and 39.8%) groups, respectively.

Testosterone treatment did not affect hatching body weight of chicks in both E1 and E2 (0.25 and 2.5 ng testosterone). However, hatchlings from eggs treated with the highest dose (25 ng) of testosterone had significantly reduced body weight as compared to control chicks (t = 2.554; $p < 0.05$; mean ± SEM for C-group 6.82 ± 0.08 g and 6.54 ± 0.08 g for T-group).

Responses measured in the tonic immobility test differed between the control and testosterone-treated group after administration of the highest concentration of testosterone (Experiment 3). Quail hatched from testosterone-treated eggs showed longer duration of TI (T = 1706; $p < 0.05$; Fig. 1A) and required fewer attempts to induce TI (T = 1216; $p < 0.05$; Fig. 1B) as compared to control birds. Treatments with both lower doses of testosterone (0.25 and 2.5 ng) did not influence TI reactions in hatchlings. However, chicks exposed to 2.5 ng of testosterone exhibited a trend to longer duration of TI (T = 452; $p = 0.093$) than control birds.

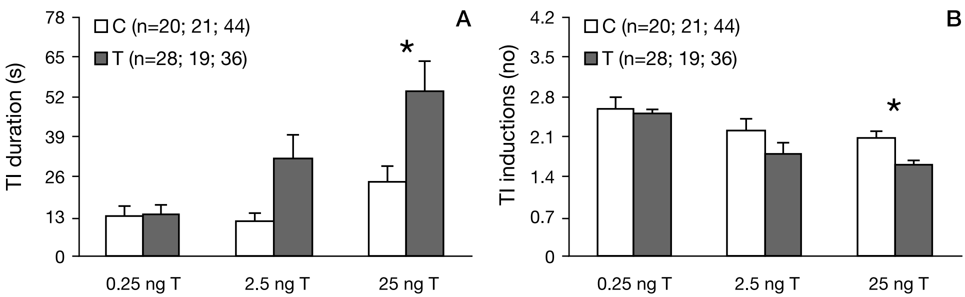

Fig. 1. Tonic immobility duration (A) and number of TI inductions (B) in 2-day-old Japanese quail hatched from control (C) and testosterone-treated (T) eggs with three different doses of testosterone. Values represent means ± S.E.M., * $p < 0.05$.

Behavioural observations in the open-field test revealed a significant effect for quail treated with experimentally increased testosterone content in eggs (Table 1). Hatchlings exposed to 2.5 ng of testosterone emitted a lower number of distress calls as compared to the control group (t = 2.285; $p < 0.05$). The main treatment effect on decreased vocalization was observed during the first 5 minutes of the open-field test (Fig. 2). Significant differences between chicks hatched from control and testosterone-treated eggs were found after the administration of both 2.5 ng (t = 2.492; $p < 0.05$) and 25 ng (t = 2.896; $p < 0.01$).

Quail hatched from eggs treated with the lowest dose of testosterone (0.25 ng) displayed both longer latency to explore (T = 77.5; $p < 0.05$) and higher defecation rates (T = 67; $p < 0.01$) as compared to the control group. Similarly, increased number of defecations (T = 67; $p < 0.01$) together with shorter latency to defecation (T = 145.5; $p < 0.05$) were found in hatchlings exposed to testosterone at the dose of 2.5 ng as compared to control birds. In Experiment 2, quail hatched from testosterone-treated eggs showed a trend to decreased locomotor activity (t = 1.925; $p = 0.069$) and exhibited a significantly higher number of jumps (T = 79; $p < 0.05$) than controls. Differences in behaviour in E3 were observed in a trend toward a longer latency to explore which was demonstrated among chicks hatched from testosterone-treated eggs (T = 84.5; $p = 0.078$) as compared to the control.

Table 1. Open-field behaviours in 1-day-old quail chicks hatched from control (C) and testosterone-treated (T) eggs with three different concentrations of testosterone (0.25; 2.5 and 25 ng).

	E1 (0.25 ng T)		E2 (2.5 ng T)		E3 (25 ng T)	
	C, n = 10	T, n = 10	C, n = 10	T, n = 11	C, n = 10	T, n = 11
Number of lines crossed	569.1 ± 73.6	640.3 ± 91.5	751.3 ± 71.6	570.4 ± 61.6	611.4 ± 50.2	533.3 ± 92.3
Number of calls	609.3 ± 59.4	569.7 ± 48.2	817.4 ± 54.0	651.9 ± 48.6 *	640.1 ± 78.7	470.6 ± 48.3
Number of jumps	72.5 ± 18.5	52.2 ± 19.7	29.1 ± 12.2	56.1 ± 11.0 *	33.5 ± 12.8	35.2 ± 13.2
Number of pecks	-	-	291.2 ± 44.0	313.1 ± 37.7	247.7 ± 44.0	259.1 ± 48.2
Number of defecations	0.3 ± 0.2	1.2 ± 0.1 **	0.4 ± 0.2	1.0 ± 0.1 *	0.9 ± 0.2	0.9 ± 0.2
Latency to vocalize (s)	2.1 ± 1.2	20.2 ± 11.8	6.6 ± 6.6	8.4 ± 5.1	0.0 ± 0.0	10.8 ± 9.5
Latency to explore (s)	13.9 ± 7.9	50.7 ± 19.2 *	14.0 ± 9.4	22.8 ± 8.8	4.3 ± 1.9	23.3 ± 13.4
Latency to defecation (s)	-	-	721.7 ± 109.3	273.4 ± 97.3 *	391.5 ± 134.9	246.4 ± 99.6

Data are presented as means ± SEM, * $p < 0.05$, ** $p < 0.01$.

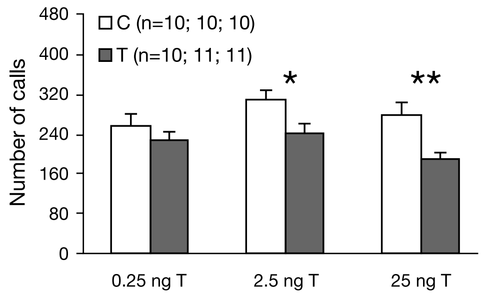

Fig. 2. Vocalization of 1-day-old Japanese quail in the first 5 minutes of the open-field test. C- control groups, T- testosterone-treated groups. Values are presented as means ± S.E.M., * $p < 0.05$, ** $p < 0.01$.

Discussion

Administration of testosterone into the egg yolk modified behavioural responses of Japanese quail hatchlings measured in two separate behavioural tests. Endogenous androgen concentrations in avian eggs are strongly species-specific and can differ both between eggs of individual females and within the order of laying sequence (Reed and Vleck 2001; Pilz et al. 2003; Groothuis et al. 2005). Recent studies in Japanese quail have determined endogenous levels of yolk testosterone in the range from 4 ng (Hayward et al. 2005) to 30 ng/g yolk (Hackl et al. 2003). Since our unpublished egg yolk testosterone determination is in agreement with these amounts, it was expected that the three tested testosterone doses (0.25 ng, 2.5 ng, and 25 ng) increased content of yolk androgens within the range of naturally occurring levels.

Results of the present study show that open-field behaviours of quail hatchlings were affected by elevated yolk testosterone content at all three concentrations. Social isolation and an unfamiliar environment are two types of stressful events which can elicit fearful behaviour among the quail that underwent open-field testing (Marin et al. 1997). In poultry species, fear can be associated with longer immobility period and inhibition of exploratory behaviour characterized by both suppressed vocalization and motor activity (Ginsburg et al. 1974; Jones et al. 1992). According to this link, an increased concentration of testosterone in egg yolk resulted in more fearful performance of hatchlings in the open-field test and followed a similar trend in all three experiments. Quail hatched from testosterone-treated eggs showed longer latency to leave the start square, decreased locomotion, enhanced defecation and lower number of distress calls as compared to control birds.

The results of the tonic immobility test were indicative of increased fearfulness of chicks exposed to elevated testosterone in egg yolk and this test would be considered analogous to an anti-predator response (Gallup 1979). Measuring tonic immobility responses is commonly used to assess an innate degree of fear since individuals with higher fearfulness display longer duration of TI and lower number of TI inductions (Jones et al. 1994; Marin et al. 2001). This pattern was observed among birds hatched from eggs treated with the highest dose of testosterone. Quail were more sensitive to induce TI and simultaneously delayed in awakening from the TI state as compared to birds in the control group.

While we examined three different concentrations of testosterone, one could expect to distinguish their effects in a dose-dependent manner. In actuality, TI duration was prolonged and number of attempts to induce TI was lower with each increasing dose of testosterone. Conversely, a dose-dependency in relation to behavioural parameters recorded in the open-field test was not observed.

The role of androgens is well-documented in the regulation of many physiological and behavioural processes and in a social context in which androgens activate and regulate aggressive and reproductive behaviour (Schlinger et al. 2001; Ros et al. 2002). Published studies in altricial birds demonstrate that experimental elevation of yolk testosterone produces positive effects on begging behaviour of offspring and on their competitive abilities (Schwabl 1996; Strasser and Schwabl 2004). In contrast to these stimulating effects, suppressed behavioural responses among 2-day-old quail hatched from testosterone-treated eggs were observed in the current study. Nonetheless, these data correspond with the hypothesis that increased testosterone content in yolk differentially influences behaviour of Japanese quail during ontogeny (Okuliarová et al. 2006). Previous works have shown that beginning the second week post-hatch, quail treated with *in ovo* testosterone injection express higher general activity in response to an unknown environment (Okuliarová et al. 2006) and are less fearful in a novel object test and test of tonic immobility (Daisley et al. 2005). Explanation of this phenomenon requires further study. It is likely that fearfulness exhibited among newly hatched chicks may affect their sensitivity to any disturbances from the environment and may impact their long-term behaviour (Heiblum et al. 1998; Richard-Yris et al. 2005). During this short post-hatch period, a critical stage for successful survival, increased fearful behaviour may represent an appropriate response to external challenges. Later, depending on prevailing environmental conditions and ontogenetic development, birds may improve their capability to cope actively with challenging situations and they turn their behavioural strategy in a "proactive" instead of "reactive" manner.

In conclusion, experimentally increased testosterone content in egg yolk elicited more intense fear-related responses of quail hatchlings during their early ontogeny. Measurable behavioural consequences with the lowest testosterone dose suggest that very slight fluctuations in yolk androgens may impact performance of future offspring. Further studies will focus on natural variability of yolk testosterone levels in eggs of precocial Japanese quail.

Účinok zvyšujúcich sa koncentrácií testosterónu vo vajci na správanie vyliahnutých mláďat prepelice japonskej

Cieľom našej práce bolo zistiť vplyv zvýšeného obsahu testosterónu vo vajci na správanie jedno- a dvoj- dňových mláďat prepelice japonskej. V rámci troch samostatných experimentov sme použili tri odlišné koncentrácie testosterónu (0,25; 2,5 a 25 ng), ktoré zvýšili obsah endogénnych androgénov vo vajci v rozsahu fyziologických hodnôt. Pri experimentálnej manipulácii bol použitý testosterón propionát riedený v objeme 20 µl olivového oleja a injekčne aplikovaný do vajcového žĺtka pred začiatkom inkubácie vajec.

Správanie vyliahnutých mláďat bolo zaznamenávané v testoch otvoreného poľa a tonickej imobility, ktoré umožňujú hodnotiť strachom podmienené reakcie testovaných zvierat.

V teste otvoreného poľa sa dôsledky zvýšeného testosterónu vo vajci prejavili pri všetkých troch aplikovaných koncentráciách a súhlasne potvrdzovali inhibíciu behaviorálnych prejavov mláďat. Prepelice vyliahnuté z testosterónom ovplyvnených vajec vykazovali dlhšiu pohybovú latenciu, zníženú lokomočnú aktivitu, zvýšenú defekáciu a nižší počet úzkostných hlasových prejavov v porovnaní s kontrolou. V teste tonickej imobility sa vplyv testosterónu manifestoval iba pri najvyššej aplikovanej koncentrácii. Mláďatá, vystavené v štádiu embrya experimentálne zvýšeným hladinám testosterónu, sa vyznačovali dlhšou tonickou imobilitou a nižším počtom pokusov potrebných na jej indukciu v porovnaní s kontrolnou skupinou.

Naše výsledky demonštrovali, že vyšší obsah testosterónu vo vajci sa prejavuje na intenzívnejších strachom podmienených reakciách mláďat prepelice japonskej. S ohľadom na predchádzajúce práce, ktoré preukázali stimulačný vplyv testosterónu vo vajci na správanie prepelíc v neskoršom vývinovom štádiu, sa zdá, že inhibičný účinok testosterónu u prekociálnych vtákov je obmedzený na obdobie krátko po vyliahnutí.

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