

Yolk testosterone and corticosterone in hierarchical follicles and laid eggs of Japanese quail exposed to long-term restraint stress

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ABSTRACT

Environmental and behavioural stimuli experienced by egg-laying female birds contribute to intra- and inter-female differences in hormones in the egg yolk with consequences for offspring development. The understanding of physiological mechanisms underlying yolk hormone deposition can aid progress in this field. In our study, we measured the concentration of testosterone and corticosterone in hierarchical follicles and egg yolks of Japanese quail in control and chronic stress conditions. Experimental females were reared under hypodynamia, a model situation for restraint stress, from day 3 to 63 days of age. For yolk hormone analysis, four largest follicles of ovarian hierarchy (F1–F4), eggs present in the oviduct and eggs laid on the day before were collected. In chronically stressed birds, yolk testosterone concentrations decreased from F2 onwards, while yolk corticosterone content was increased from the beginning to the end of egg formation. The follicular profile of hormones suggested testosterone transfer into the yolk directly from granulosa and theca cells, with the highest accumulation during a period 48–72 h before laying the egg. Yolk corticosterone was accumulated from maternal plasma preferentially in early stages of follicular development under control conditions and also in last stages of egg formation under stress conditions. These specific patterns of hormone deposition indicate periods when stimuli experienced by female can substantially modify hormonal content of eggs. Lower testosterone and increased corticosterone yolk concentrations in stressed quail may represent signals mediating information about adverse environmental conditions from the mother to progeny.

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1. Introduction

Avian eggs contain a variety of maternal hormones including steroids (Schwabl, 1993), derivatives of amino acids (Wilson and McNabb, 1997) and peptides (De Pablo et al., 1982). Among these substances yolk steroids have attracted the greatest interest since they are considered to represent one of the epigenetic factors that can modify gene expression in response to the maternal environment (Horton, 2005). In addition, the organizational action of steroid hormones is manifested mainly during the early ontogeny, the critical period for development of neural circuits and behaviour. The embryonic exposure to maternal hormones may modify not only early development of young, but also their adult behavioural phenotype (Eising et al., 2006; Partecke and Schwabl, 2008) and prepare them to cope more effectively with their actual environment (Mousseau and Fox, 1998).

Experimental research on the role of maternal hormones has been carried out in different avian species and has focused predominantly on androgens in the egg yolk (Groothuis et al., 2005). These data revealed apparent differences in yolk androgen concentrations among individual females and species dependent intra-individual differences between eggs of a single clutch reflecting their laying order (Reed and Vleck, 2001; Groothuis and Schwabl, 2002; Pilz et al., 2003). The inter-female variability in deposition of androgens into eggs was shown to be related to numerous external and internal factors including breeding density (Schwabl, 1997; Pilz and Smith, 2004), social hierarchy (Tanvez et al., 2008), food availability (Gasparini et al., 2007), ectoparasitic exposure (Tschirren et al., 2004) and genetic variation (Gil and Faure, 2007).

A clear impact of maternal androgens, especially yolk testosterone, on the development and survival of offspring was shown by administration of physiological doses of hormones into eggs (reviewed in Groothuis et al., 2005). In Japanese quail, increased testosterone content in the egg influenced behaviour in the offspring in a proactive way since young birds exhibited low levels of fear-related responses (Daisley et al., 2005) and increased activity in an open-field test (Okuliarová et al., 2006). The proactive effect

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of testosterone was seen from day 9 of posthatch development while 2-day-old chicks exhibited higher level of fear (Okuliarová et al., 2007).

Corticosterone is the dominant plasma glucocorticoid in birds and concentrations in avian eggs were positively affected by an experimental increase of this hormone in maternal circulation (Hayward and Wingfield, 2004; Love et al., 2005) as well as by simulating stress conditions during egg laying (Saino et al., 2005). In Japanese quail, elevated plasma corticosterone resulted in increased yolk corticosterone content in eggs laid 7 days after experimental manipulation (Hayward and Wingfield, 2004). In the only study with an analytical validation (Rettenbacher et al., 2005) immunoreactive corticosterone was not detected in chicken eggs and the authors propose that the reported corticosterone concentrations are mainly cross-reactions with other steroids that are found in the yolk in abundant concentrations.

Published data suggest that maternal hormones may influence behavioural phenotype, personality differences and possibly the fitness of offspring in fluctuating environment. Nonetheless, the physiological mechanisms of maternal hormone deposition into eggs need to be better understood to appreciate the adaptive value of maternal “investment”. These mechanisms probably differ depending on the type of hormone and where it is synthesised (Groothuis and Schwabl, 2008). Sex steroids are produced in theca and granulosa cells of ovarian follicles (Huang and Nalbandov, 1979). On the other hand, other groups of maternal hormones found in the egg yolk, including corticosterone, are synthesised outside the ovary.

In our study, we measured yolk testosterone and corticosterone concentrations in hierarchical follicles and laid eggs of Japanese quail exposed to chronic restraint stress. Quail were reared under hypodynamia, which represents a model situation generally used to simulate weightlessness and microgravity environment experienced during space flights (Sulzman, 1996). Under these conditions quail are challenged by restraint stress since the long-term hypodynamia increases plasma corticosterone levels and changes neurohumoral activity of adult female quail (Juráni et al., 1983). First, we tested the prediction that yolk testosterone will decrease and yolk corticosterone will increase in chronically stressed birds. The second aim of our study was to determine a pattern of maternal hormone deposition into the yolk in relation to follicular maturation under both control and experimental conditions. This methodological approach of yolk hormone analysis from early follicular stages to the matured egg may bring an insight into physiological mechanisms how and when testosterone and corticosterone are transferred into the yolk. We predict different follicular profiles for these hormones which can reflect different ways by which they pass into the yolk.

2. Materials and methods

2.1. Animals and experimental design

Female Japanese quail of laying strain were hatched and reared in a breeding colony at the Institute of Animal Biochemistry and Genetics, Slovak Academy of Sciences, Ivanka pri Dunaji, Slovak Republic. Experimental females were exposed to long-term hypodynamia from day 3 after hatching till 63 days of age. Briefly, this procedure was performed by placing animals in special individual slings suspended by a flexible wire apparatus in such a way that their legs cannot touch the floor (Škrobánek et al., 2004).

Quail were housed under continuous lighting and they were provided with a commercial starter mash for young turkeys and water *ad libitum*. Temperature was maintained at 35–36 °C for the first week after hatching and then it was gradually reduced to about 20 °C during the next four weeks. Both restrained and

control groups were held in the same room in separate rearing boxes.

Control females reached their sexual maturity earlier than hypodynamia reared quail, but laying rate did not differ between both groups (Škrobánek et al., 2008). At the age of 63 days, seven females of each group were killed by decapitation between 8:00 and 9:30 h. Their ovaries were dissected and four largest follicles of follicular hierarchy (F4 < F3 < F2 < F1) as well as shelled eggs present in oviduct (EO) were collected. In addition, eggs laid on the day before decapitation (EL) were collected and included in yolk steroid analysis. All follicles and separated yolks were weighed and stored at –20 °C.

The care and use of animals were in accordance with laws and regulations of Slovak Republic and approved by Ethical Committee of the Institute of Animal Biochemistry and Genetics, Slovak Academy of Sciences, Ivanka pri Dunaji.

2.2. Yolk steroid extraction and assays

Testosterone in ovarian follicles and egg yolks was extracted from a small amount of yolk (40–45 mg) which was withdrawn by spatula from the inside of follicles and from the homogenised egg yolks, respectively. Samples were diluted in 500 µl of deionised water and vortexed with addition of two glass beads for 3 min. Approximately 1500 dpm of [³H]-testosterone was added to each sample for individual recovery calculation. Samples were equilibrated overnight at 4 °C. Thereafter they were applied on solid phase columns filled with Extrelut NT (Merck, Darmstadt, Germany) and extracted twice with 2 ml and once with 1 ml of diethyl-/petroleum ether (7:3). Following evaporation under a stream of nitrogen, dried extracts were reconstituted in 300 µl of phosphate buffer (pH = 7.5) and frozen until measurement by radioimmunoassay validated for bird plasma in our laboratory (Zeman et al., 1986). Average recovery for testosterone was 58.6 ± 0.7%.

Concentrations of yolk testosterone were measured in 20 µl aliquots of the extract using [1,2,6,7-³H]-testosterone (Amersham Biosciences, UK, specific activity 3.52 TBq/mmol) and a specific antibody generated in rabbits against testosterone-3-(carboxymethyl) oxime bovine serum albumin conjugate (obtained from Dr. D. Pichova, Czech Republic). Cross-reactivity of antiserum with other relevant steroids was 9.6% with 5α-dihydrotestosterone, 0.1% with androstenediol, 0.04% with androsterone and less than 0.01% with other steroids. Bound and free fractions were separated by dextran-coated charcoal and centrifuged at 3000g at –20 °C for 10 min. All samples were run in a single assay. The intra-assay coefficient of variation calculated for pooled quail plasma was 10.8%. Minimal concentrations of testosterone in the egg yolk were detected at the level 0.93 pg per tube.

As the F4 follicles were completely used for testosterone analysis, corticosterone was analysed only in the three largest follicles (F3, F2 and F1) and in yolks of eggs in oviduct and laid eggs. In brief, 0.15 g yolk of each follicle was diluted in 600 µl of distilled water, vortexed for 30 s and frozen overnight. Next day, 3 ml of 100% methanol was added, samples were shaken for 30 min and frozen overnight. After centrifugation, 1 ml of the supernatant was evaporated under a stream of nitrogen and then resuspended in 500 µl of assay buffer. Corticosterone concentrations were determined in 50 µl duplicates by a corticosterone enzyme immunoassay described by Palme and Möstl (1997). The sensitivity of the assay was 3.5 pg per tube. The intra- and inter-assay coefficients of variation were 11% and 16%, respectively.

2.3. Statistical analysis

Data were analysed using General Linear Model (GLM) with treatment (chronic stress and control), developmental stage of

egg and the interaction between them defined as fixed factors. Female identity was included as a random effect nested within treatment group. The analysis of yolk hormone levels was calculated for the concentration of testosterone and corticosterone as well as for the total yolk testosterone and corticosterone content (ng per whole yolk), respectively. All data except those for yolk testosterone content were square root transformed to fit normal distribution. Post hoc comparison was performed on the base of LSD tests.

3. Results

3.1. Weight of follicles and egg yolks

Statistical analysis of ovarian follicle and egg yolk weight revealed significant effects of experimental treatment ($F_{(1,50)} = 5.9092$; $p < 0.05$), developmental stage of egg ($F_{(5,50)} = 114.5832$; $p < 0.001$), no interaction between both factors ($F_{(5,50)} = 0.8193$; $p = 0.542$) and significant differences among individual females ($F_{(12,50)} = 3.0833$; $p < 0.01$). Weight of follicles and yolks decreased in quail under chronic restraint stress compared with control (Fig. 1). During follicular maturation the weight was linearly increasing from the F4 follicle to the stage EO, which did not differ in comparison with the yolk of laid eggs (EL).

3.2. Testosterone concentrations and total testosterone content

Concentrations of yolk testosterone were significantly influenced by both experimental treatment ($F_{(1,51)} = 5.4586$; $p < 0.05$) and developmental stage of egg ($F_{(5,51)} = 7.1456$; $p < 0.001$). Although the analysis revealed no significant interaction between these factors ($F_{(5,51)} = 1.6716$; $p = 0.158$), post hoc comparison showed that long-term restraint stress caused lower concentrations of testosterone in the F2 follicle ($p < 0.01$) compared with control females (Fig. 2A). The same trend was seen in the F1 follicle ($p = 0.071$), but no differences were found in follicles F3 and F4. In addition, quail reared in hypodynamic conditions laid eggs (EL) with decreased testosterone concentrations compared with the control ($p < 0.05$). In the follicular hierarchy of control females, testosterone gradually increased from the F4 follicle to the maximum concentration measured in the F2 follicle ($p < 0.001$). Thereafter testosterone concentrations decreased from the F2 to the F1 follicle, EO and EL ($p < 0.01$). This pattern was less pronounced, but still apparent in the chronically stressed group with the highest testosterone in follicles F3 and F2. An increase was found between the F4

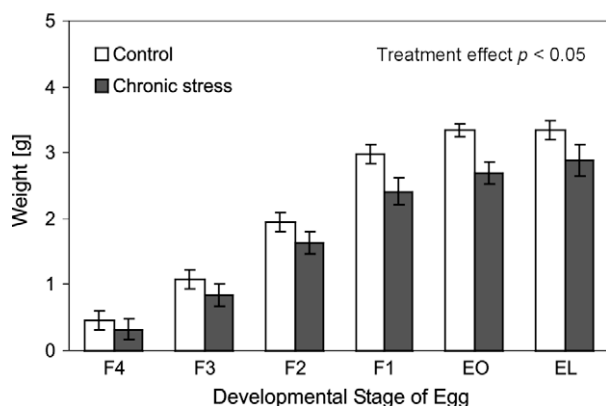


Fig. 1. Weight of hierarchical follicles (F4, F3, F2 and F1), yolks of eggs found in oviduct (EO) and eggs laid before decapitation (EL). Treatment effect indicates differences between control quail ($n = 4$ for follicles F4, $n = 7$ for follicles F3, F2 and F1, $n = 6$ for the EO and EL) and quail reared under chronic restraint stress ($n = 6$ for follicles F4, $n = 7$ for follicles F3, F2 and F1, $n = 5$ for the EO and EL). Values are presented as means \pm SEM.

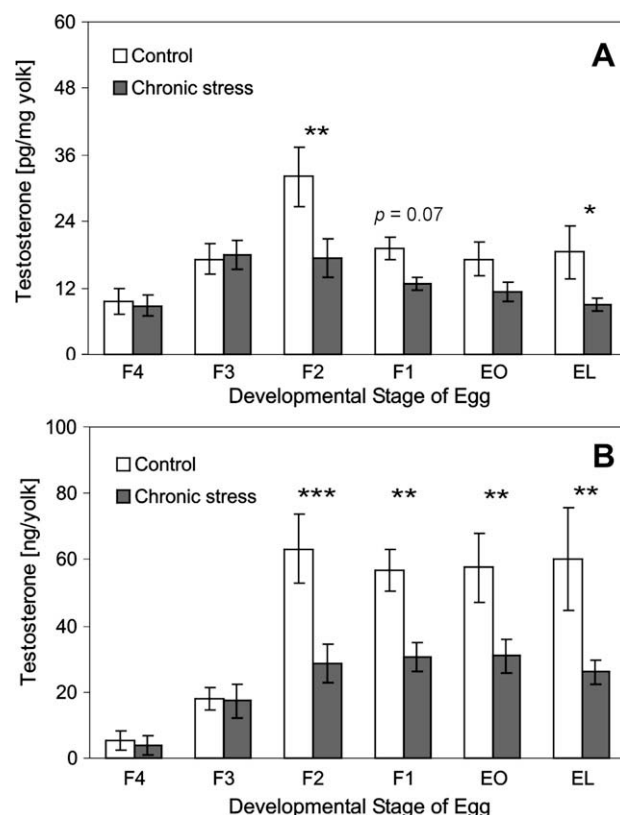


Fig. 2. Testosterone concentrations (A) and total testosterone content (B) in hierarchical follicles (F4, F3, F2 and F1), yolks of eggs found in oviduct (EO) and eggs laid before decapitation (EL). LSD post hoc comparison between control quail ($n = 4$ for follicles F4, $n = 7$ for follicles F3, F2 and F1, $n = 6$ for the EO and EL) and quail reared under chronic restraint stress ($n = 6$ – 7 for follicles F4, $n = 7$ for follicles F3, F2 and F1, $n = 5$ for the EO and EL) is marked at the level of significance * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$. Values are presented as means \pm SEM.

follicle and both F3 ($p < 0.01$) and F2 ($p < 0.01$) follicles and a decrease was found between the F3 follicle and EL ($p < 0.05$) as well as between the F2 follicle and EL ($p < 0.05$).

Results for total testosterone content showed significant differences between experimental treatments ($F_{(1,50)} = 9.1611$; $p < 0.01$), developmental stages of egg ($F_{(5,50)} = 11.8122$; $p < 0.001$) and their interaction ($F_{(5,50)} = 2.5506$; $p < 0.05$). In the restraint stressed group, yolk testosterone content was lower in follicles F2 ($p < 0.001$) and F1 ($p < 0.01$), in the EO ($p < 0.01$) and EL ($p < 0.01$) compared with the control group (Fig. 2B). No differences between both groups were found in follicles F3 and F4. Total testosterone content increased with follicular maturation from the F4 to the F2 follicle, while in the next stages it remained constant. Significant differences were recorded in follicles F4 and F3 compared with F2, F1, EO and EL ($p < 0.001$) in the control group and in the F4 follicle compared with F2, F1, EO ($p < 0.01$) and EL ($p < 0.05$) in the chronically stressed group.

Variability between individual females was determined by the significant effect of female identity on both testosterone concentrations ($F_{(12,51)} = 2.0575$; $p < 0.05$) and total testosterone content ($F_{(12,50)} = 2.5662$; $p < 0.01$).

3.3. Corticosterone concentrations and total corticosterone content

Concentrations of yolk corticosterone were significantly influenced by both experimental treatment ($F_{(1,36)} = 16.1694$; $p < 0.01$) and developmental stage of egg ($F_{(4,36)} = 3.9739$; $p < 0.01$), while there was no significant interaction between these factors ($F_{(4,36)} = 1.3153$; $p = 0.2827$). Females reared under long-term restraint

stress displayed higher corticosterone concentrations in hierarchical follicles (F3, F2, F1) as well as in ovulated yolks of the EO and EL compared with the control group (Fig. 3A). In the follicular hierarchy, corticosterone concentrations were lower in the F3 follicle than F1 ($p < 0.05$), EO ($p < 0.01$) and EL ($p < 0.01$) in control birds. In the chronically stressed group, there was only a difference in the F3 follicle compared with EL ($p < 0.05$).

Total corticosterone content of ovarian follicles and egg yolks increased in females reared under restraint stress conditions compared with control females ($F_{(1,36)} = 17.8651$; $p < 0.001$; Fig. 3B). There was no significant effect of developmental stage of eggs ($F_{(4,36)} = 0.3893$; $p = 0.815$) and no interaction between these factors ($F_{(4,36)} = 2.1515$; $p = 0.094$).

The effect of female identity on corticosterone concentrations ($F_{(12,36)} = 1.8936$; $p = 0.069$) and total corticosterone content ($F_{(12,36)} = 1.6307$; $p = 0.126$) was not significant.

4. Discussion

In the present study, we demonstrated that the chronic restraint stress resulted in reduced testosterone and increased corticosterone levels in the yolk of hierarchical and ovulated follicles and laid eggs of Japanese quail. Both steroid hormones have been analysed in the egg yolk of domestic and wild avian species but only in a limited number of experimental studies have these hormones been simultaneously measured (Schwabl, 1993; Hayward et al., 2005; Pike and Petrie, 2005; Navara et al., 2006).

An exposure to a stressful stimulus activates the hypothalamic–pituitary–adrenal axis, which in turn induces a series of physiological and behavioural changes to restore homeostasis (Sapolsky et al., 2000). In birds, the main outcome of this pathway is elevated plasma corticosterone (Silverin et al., 1997; Romero and Reed, 2005). Previous experiments using corticosterone implants in female Japanese quail demonstrated that increased maternal glucocorticoids can pass into the yolk during egg formation (Hayward and Wingfield, 2004). Our study extends this to show increased endogenous corticosterone concentrations in the egg yolk of quail exposed to long-term stress conditions. Another study showed that female peafowl (*Pavo cristatus*) laid eggs with higher yolk corticosterone and a tendency to lower yolk testosterone levels, when they were exposed to potentially stressful situation and had to mate to males with experimentally reduced attractiveness (Pike and Petrie, 2005).

Several factors negatively influence testosterone concentrations in the egg yolk of different avian species. Decreased deposition of yolk androgens was observed as a consequence of early developmental stress during nestling period in zebra finches (*Taeniopygia guttata*) (Gil et al., 2004) and after the challenge to the immune system in female house martins (*Delichon urbica*) (Gil et al., 2006).

Variable physiological and behavioural effects of maternal hormones on embryonic and postnatal development of offspring are well documented (Groothuis et al., 2005). In Japanese quail, experimentally increased yolk testosterone content resulted in a proactive behavioural strategy of young birds (Daisley et al., 2005; Okuliarová et al., 2006), while in domestic chicks, experimentally elevated corticosterone concentrations in egg evoked increased fear-related responses of young during behavioural tests (Janczak et al., 2006). Therefore, decreased yolk testosterone and increased corticosterone concentrations together with lower weight of follicles and egg yolks in chronically stressed quail suggest possible implications for offspring's phenotype.

Our study analysed testosterone and corticosterone concentrations in hierarchical follicles and yolk of forming eggs. We found differential changes in both hormones during follicular maturation corresponding with the different ways they are transferred into the egg yolk. Testosterone and other sex steroids are mainly produced by granulosa and theca cells, which are organised in specialised layers and constitute the wall of ovarian follicles (Huang and Nalbandov, 1979). While these cells fully enclose the yolk content, they can affect the amount of yolk androgens either directly or indirectly through the blood circulation. Thus, several physiological mechanisms regulating the deposition of yolk androgens are possible (reviewed in Groothuis and Schwabl, 2008). In our study, an increase of testosterone concentrations from the F4 to the F2 follicle was followed by a decline in the largest F1 follicle and this pattern clearly corresponds to the production of testosterone in chicken granulosa and theca cells (Bahr et al., 1983). Moreover, the testosterone profile in hierarchical follicles is parallel to concentric distribution of sex steroid hormones within the egg yolk displaying the maximum of testosterone concentrations in the middle and low levels in the outer layer of the yolk (Lipar et al., 1999; Möstl et al., 2001; Hackl et al., 2003). In the painted turtle (*Chrysemys picta*), a similar pattern for yolk testosterone concentrations within preovulatory follicles was described (Elf et al., 2002). The parallelism between testosterone profile in hierarchical follicles and characteristic distribution of sex steroids in the yolk suggests that the direct transfer of testosterone from granulosa and theca layers contributes preferentially to the yolk testosterone content.

Our results showed differences in testosterone concentrations between control and chronically stressed quail first appeared in the F2 follicle. The F2 follicle is known for its higher sensitivity to the luteinizing hormone (LH) stimulated androgen production

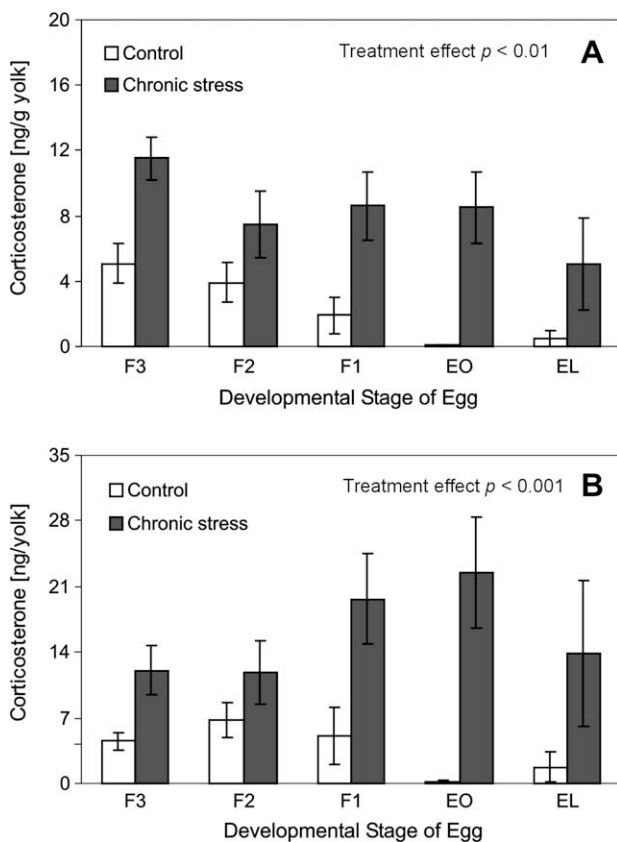


Fig. 3. Corticosterone concentrations (A) and total corticosterone content (B) in hierarchical follicles (F3, F2 and F1), yolks of eggs found in oviduct (EO) and eggs laid before decapitation (EL). Treatment effect indicates differences between control quail ($n = 6$ for follicles F3, $n = 7$ for follicles F2 and F1, $n = 5$ for the EO, $n = 4$ for the EL) and quail reared under chronic restraint stress ($n = 5$ for follicles F3, EO and EL, $n = 7$ for follicles F2 and F1). Values are presented as means \pm SEM.

compared to F1 (Robinson and Etches, 1986). Thus, exposure to chronic stress may either change the receptor sensitivity of follicles or modify the LH production on the central hypothalamic–pituitary level. These data indicate a critical period for yolk testosterone accumulation in the egg yolk 48–72 h before laying the egg. Environmental and behavioural stimuli experienced by female during this time might be crucial for testosterone content in the egg and this interval should be taken into account when variability of yolk testosterone deposition is considered in relation to individual female responses to external and internal factors.

Since corticosterone synthesis is located in the adrenal glands, its transfer into the yolk must occur from the circulation of female. We found elevated corticosterone in all examined follicular stages after chronic exposure to restraint stress compared with quail reared in control conditions. Corticosterone concentrations decreased during follicular maturation being the highest in the F3 follicle and the lowest in the yolk of laid eggs. The decreasing pattern from the F3 to F1 follicle was clear in control animals, in which we found very low corticosterone concentrations in yolks of shelled eggs similar to those reported in other studies (Schwabl, 1993; Hayward and Wingfield, 2004). These findings suggest that corticosterone enters the yolk preferentially in early follicular stages and further accumulation in the yolk is limited under control conditions. However, in stressed quail, the corticosterone content continued to increase during last stages of egg formation suggesting enhanced transfer from maternal plasma under chronic stress. While the corticosterone content per whole yolk did not vary between individual follicles in control birds, we can speculate that corticosterone diffuses from the yolk into the egg white. Indeed, studies in hen and barn swallow demonstrated that maternal stress resulted in increased corticosterone levels in albumen (Downing and Bryden, 2002; Saino et al., 2005). An alternative explanation could be metabolism or degradation of the hormone during egg formation.

Although we did not perform an analytical validation to ensure that we really measured corticosterone, we conclude that measured concentrations cannot be caused by progesterone cross-reactions, because the pattern in follicles over maturation does not match the increasing production rate of gestagens in large follicles (Bahr et al., 1983). Furthermore, in quail exposed to hypodynamia plasma progesterone concentrations did not rise, but decreased (Škrobánek et al., 2008).

In conclusion, our study is the first report on testosterone and corticosterone concentrations in hierarchical follicles and yolks of pre-laid eggs. Data describe different profiles for deposition of both hormones into the yolk during follicular maturation under control and chronic stress conditions. Testosterone is deposited into the yolk predominantly by direct way from granulosa and theca cells with the maximum rate during a period 48–72 h before laying the egg. The ability of female to respond to social and environmental stimuli by testosterone biosynthesis can increase during this period and substantially modify androgen content in her eggs. By contrast, corticosterone is transferred into the yolk especially during early stages of follicular development. In stressed quail, the substantial increase of corticosterone levels was also seen during later stages of egg formation, indicating that its transfer from maternal circulation can be reinforced under challenged situations. Yolk corticosterone concentrations in laid eggs were lower than the amount deposited into follicles. Therefore, both deposition and further metabolism or excretion from the yolk may determine the total content of hormone found in the egg yolk. Moreover, decreased yolk testosterone and increased yolk corticosterone concentrations together with lower weight of yolk in chronically stressed quail may represent maternal effects, though which development of young can be adjusted for surviving in unfavourable conditions expected after hatching. A better understanding of

mechanisms of yolk hormone deposition creates a background for behavioural and ecological studies concerning intra- and inter-female variability in yolk hormone concentrations.

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