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Variable effects of elevated egg yolk testosterone on different arms of the immune system in young quail

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1. Introduction

Maternal hormones can serve as a tool for the transfer of information about the actual environment from the parental generation to the offspring (Dufty et al., 2002). Modifications of phenotype caused by this transfer can contribute to faster adaptations of the offspring to rapidly changing environmental conditions (Groothuis et al., 2005). In birds, the effects of egg yolk testosterone (T) on progeny have been investigated since its positive influence on the growth of the altricial canary was described (Schwabl, 1996). Other research groups confirmed stimulatory effects of maternal T on postnatal growth (Muriel et al., 2013; Okuliarova et al., 2011b) as well as on the development of proactive behavior (Müller et al., 2012, 2009; Noguera et al., 2013; Okuliarova et al., 2006). Yolk T promoted faster growth, while more proactive behavior should provide a selective advantage in terms of competitive abilities (Müller et al., 2012), occupying territories (Müller et al., 2009), obtaining food resources (Eising and Groothuis, 2003; Pilz et al., 2004) or capabilities to obtain and maintain mating partners (Bonisoli-Alquati et al., 2011).

On the other hand, possible disadvantages of increased maternal T may include enhanced metabolic rate (Ruuskanen et al., 2013), increased energy expenditure (Tobler et al., 2007), impaired oxidative damage repair (Treidel et al., 2013) and increased mortality (Sockman and Schwabl, 2000). Some of these costs are connected with activation of the immune system. It is often predicted that high maternal T can lead to suppression of the immune systems; this prediction is based on results obtained in adult males (Casto et al., 2001; Peluc et al., 2012). In some studies, experimentally increased egg yolk T caused an inhibition of the immune system in a similar way as in adult animals (Müller et al., 2005; Sandell et al., 2009). However, other studies showed no effects (Pitala et al., 2009; Rubolini et al., 2006a; Ruuskanen et al., 2013) or even reported a positive influence of maternal T on immune responsiveness (Navara et al., 2006; Tobler et al., 2010).

These contradictory results might be partially explained by a dose dependent manner of maternal T action and different experimental models. Moreover, maternal T can affect differently various parts of the immune system (Clairardin et al., 2011; Muriel et al., 2017; Tobler et al., 2010). The immune system is organized into complex hierarchical levels. The multilevel regulation serves as a back-up mechanism to ensure adequate protection of the organism. Pro-inflammatory mediators (including interleukin-6 and chemokine K203) belong to signals synchronizing the immune system. They are produced during the acute phase response induced by various antigens such as lipopolysaccharide (LPS) (Leshchinsky and Klasing, 2003; Sick et al., 2000). Moreover, these cytokines previously showed a capacity to be influenced by sex steroid hormones (Kaňková et al., 2016).

In the current study, we integrated two experimental approaches in order to elucidate the effects of maternal T on different parts of immune responsiveness. We used Japanese quail (*Coturnix japonica*) genetically selected for high (HET) and low (LET) egg yolk T. These lines represent two distinct populations of birds with well-defined individual variation in maternal T levels within the physiological limits for given species (Okuliarova et al., 2014). In our study, the genetically determined levels of maternal T were further increased by exogenous *in ovo* T treatment. In this way, we established four groups differing in egg T content and possibly also with different sensitivity of tissues to this hormone. This approach allows us to observe the effects of increased egg yolk T levels on immune parameters of offspring. Furthermore, this approach can enable us to verify whether the experimental increase of egg yolk T mimics the effects of genetic selection for high egg yolk T.

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We focused on immune parameters which are likely to respond to immunological stimulation even in the early stages of development. The phytohemagglutinine (PHA) test is routinely used in eco-immunological studies for evaluation of the cell-mediated immune response (CMI) (Tella et al., 2008). To overcome problems with the immature humoral immune response, we used LPS as a T-cell independent antigen. This antigen is able to elicit a non-specific antibody response without a need to involve T-cells in activation of antibody production (Mosier and Subbarao, 1982). During the acute phase response, we chose to analyze two pro-inflammatory cytokines (inrleukin-6 and chemokine K203), which respond to LPS stimulation and showed a potential to be influenced by sex steroid hormones (Kaňková et al., 2016). Moreover, we determined the heterophil/lymphocyte ratio as a parameter linking the immune and the stress response in birds (Maxwell, 1993).

2. Material and methods

2.1. Animals and housing

Birds used in these experiments originated from the eighth generation of Japanese quail selected for high and low egg yolk T deposition (Okuliarova et al., 2011a). Egg yolk T concentrations differed significantly between lines with average values 5.7 ± 0.2 and 18.1 ± 1.0 pg/mg for the LET and HET line, respectively. Lines were bred at the Institute of Animal Biochemistry and Genetics, Slovak Academy of Sciences (IABG), Ivanka pri Dunaji, Slovak Republic. Within eight days we collected 113 and 101 eggs from the LET and HET females, respectively. Prior to the incubation, one group of eggs from each line (56 and 46 for the LET and HET eggs, respectively) was injected with 50 ng of T propionate (Agovirin Leciva, CR) per egg dissolved in 20 μl of sterile olive oil. Control eggs received 20 μl of sterile olive oil. Eggs were incubated in the forced draught incubator (BIOSKA, Sedlcany, Czech Republic) under standard conditions (temperature 37.5 ± 0.2 °C; relative humidity 50–60%; with the automatic rotation of eggs every hour). Hatchability was 61.1% and 60.4% for the LET and HET line, respectively. Hatchlings were marked with a unique combination of colored rings on their legs. They were placed into four rearing cages according to the line and treatment group. Chicks were kept under constant light and controlled temperature (gradual decrease from 37–35 °C after hatching to 27–28 °C at the end of the experiment). Food (a starter feeding mash) and water were provided *ad libitum*. After hatching, we randomly selected 80 birds; 20 quail for each line and treatment. The half of birds was tested for the cell-mediated immune response after PHA stimulation. The second half of the quails received LPS to stimulate the T-cell independent antibody response and acute phase response. The study was conducted in accordance with the laws and regulations of the Slovak Republic and approved by the Ethical Committee of IABG and State Veterinary Authority for Slovak Republic.

2.2. Stimulation with phytohemagglutinin

Phytohemagglutinin was used to stimulate a cell-mediated immune response at the age of 20 days (n = 10 birds per group). The right wing web was cleaned from feathers and its initial thickens was measured with a micrometer (Vogel, Germany, with the precision of 0.001 mm; five measurements per wing). After the basal measurement, the wing web was subcutaneously injected with PHA (Sigma-Aldrich, Saint Louis, MO, USA) in a dose of 100 μg of PHA dissolved in 20 μl of phosphate buffered saline (PBS). After 24 h, the wing web thickness was re-measured. We calculated the CMI response as the difference between the basal and stimulated wing web thickens.

2.3. Stimulation with lipopolysaccharide

In order to stimulate the humoral immune response, chicks from all four groups (n = 10 animals per group) were intraperitoneally injected with LPS (Sigma-Aldrich, Saint Louis, MO, USA; 1.5 mg per kg of body mass) dissolved in 100 μl of sterile PBS at the age of 19 days. At this age quail should be immunocompetent (Mast and Goddeeris, 1999). Blood samples were collected from the wing vein into heparinized test tubes before as well as two and eight days after the initial LPS injection. In order to measure the acute phase response, we repeatedly injected all chicks with the same dose of LPS (1.5 mg per kg of body mass) at the age of 27 days. Birds were sacrificed three hours after the second LPS injection. Blood was collected and spleen was snap frozen in liquid nitrogen and stored at −80 °C until gene expression measurement.

2.4. Heterophil/Lymphocyte ratio

Blood smears were prepared from approximately 5 μL of fresh blood, in duplicates. Dried blood smears were fixed and stained by the panoptic method of Pappenheim, previously validated in our laboratory (Kankova et al., 2012). The heterophil/lymphocyte (He/Ly) ratio was determined by differential counting of white blood cells (WBC; heterophils, lymphocytes, eosinophils, monocytes and basophils) per 200 leukocytes on each slide. Blood smears were prepared from the blood samples collected before and 8 days after the first LPS stimulation. The last sampling took place three hours after the second LPS stimulation during the acute phase response. Afterwards, blood was centrifuged (2500g, 10 min, 4 °C) and plasma was stored at −20 °C for plasma antibody examination.

2.5. Total plasma antibody levels

Total plasma antibody levels were determined by enzyme-linked immunosorbent assay (ELISA) as described previously (Kankova et al., 2014). The 96-well-plate was coated with 100 μl anti chicken IgG (Sigma-Aldrich, Saint Louis, MO, USA) diluted 1:800 with carbonate buffer (pH 9.6) and incubated at 4 °C until the following day. After a wash (3 × 100 μl of PBS-0.05% Tween 20), plates were blocked with 2.5% milk powder (diluted in PBS) and incubated for 2 h at 37 °C. The next wash was followed by adding 100 μl of sample or serial dilutions of chicken IgY standard in a range between 6.25 $ng.ml^{-1}$ and 200 $ng.ml^{-1}$ (Promega, Madison, WI, USA). The plate with samples was incubated for 1 h at 37 °C. After incubation and washing (3 × 100 μl of TBS-0.05% Tween 20), 100 μl of alkaline-phosphatase-conjugated anti-chicken IgY (Sigma-Aldrich, Saint Louis, MO, USA) diluted in 0.2% milk powder in TBS (1:1000) was added. Following incubation and washing, a substrate buffer with p-nitrophenyl phosphate (Merck, Darmstadt, Germany) was added to each well and incubated at room temperature in the dark for 20 min. The reaction was stopped by 1 $mol.l^{-1}$ NaOH and absorbance was measured at a wavelength of 405 nm in an ELISA reader (Bio-Tek Instruments, Winooski, VT, USA).

2.6. Gene expression

The spleen was homogenized in 1 ml of TRI Reagent (Molecular Research Center, INC., Cincinnati, OH, USA) by the FastPrep-2 (MP Biomedicals, Santa Ana, CA, USA; 6,0 $m.s^{-1}$; 40 s). Chloroform extraction was used for the isolation of mRNA from the homogenate. Concentration and purity of isolated RNA were determined with a NanoDrop Spectrophotometer (Peqlab Biotechnologie GmbH, Erlangen, Germany). The integrity of RNA was tested by gel electrophoresis. Isolated mRNA was treated with DNase I (Thermo Fisher Scientific Baltics UAB, Vilnius, Lithuania). After-

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