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The effect of maternal immunization on female oxidative status, yolk antioxidants and offspring survival in a songbird

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ABSTRACT

Immune defense involves inflammatory reactions in which immune cells produce reactive oxygen species (ROS) to fight pathogens. ROS may however cause damage to the host if they are not balanced by antioxidant defenses. Therefore, one should expect individuals undergoing an immune reaction to use antioxidants to prevent oxidative stress. Antioxidants are vital compounds that provide important protection against oxidative damage of embryos and newly hatched chicks. Thus, during egg laying a female that contracted an infection may face a trade-off between the allocation of antioxidants into self-maintenance and into her offspring via the eggs. In our study we investigated whether immunized females face this trade-off and consequently modify the antioxidant allocation into the eggs and whether this allocation affects offspring performance. We injected female zebra finches (*Taeniopygia guttata*) with lipopolysaccharide prior to egg laying while some females were left unimmunized. We removed the second egg of each clutch, while we allowed the other eggs to hatch. We assessed oxidative stress in females 24 h after immunization, yolk antioxidant capacity of the second egg of the clutch and survival success of the offspring until adulthood. Compared to controls, immunized females had higher oxidative damage, but similar plasma non-enzymatic antioxidant levels. The treatment did not affect yolk antioxidants, clutch size, laying date and offspring survival. However, we found a positive correlation between yolk antioxidant capacity and offspring survival, irrespective of the treatment. Our study suggests that our immune challenge may not have changed female strategy of antioxidant allocation between self-maintenance and offspring survival.

1. Introduction

Animals are critically reliant on the immune system, which protects them against parasites and pathogens (Bonneaud et al., 2003). Reactive oxygen species (ROS) constitute an important part of an immune defense (Sorci and Faivre, 2009). ROS serve to kill pathogens, but their overproduction may cause oxidative damage to the host if they are not counterbalanced by the antioxidant defenses (Sorci and Faivre, 2009; Costantini and Møller, 2009). Oxidative stress results from a disturbance in the balance between pro-oxidants and antioxidants, leading to oxidative damage to proteins, lipids and nucleic acids (Halliwell and Gutteridge, 2007).

A central idea of the so-called immuno-oxidative ecology is that oxidative stress may provide a currency for quantifying the physiological costs of the immune activation, given the negative impact of oxidative stress on growth, reproduction and senescence (Hasselquist and Nilsson, 2012; Costantini, 2014). These costs may become particularly evident during demanding periods of an individual's life cycle, such as during the breeding season, when individuals have to tradeoff the investment in self-maintenance against that in reproduction. For example, Christe et al. (2012) found that increased parental effort reduces antioxidant protection and increases malaria infection in male great tits (*Parus major*). Egg production is another demanding reproductive activity, which may result in several costs to the laying female. Females transfer into the eggs substances (antibodies, hormones, lysozyme and antioxidants) that are vital for offspring development (Williams, 1994). Given that resources occur in limited supply, their transfer into the eggs would result in lower amount of resources available for the female to sustain her self-maintenance functions (Stearns, 1992). For example, females experimentally forced to produce extra eggs suffered more oxidative stress than females that were not forced to do so (Travers et al., 2010). The oxidative cost of egg production might, however, be dependent on the female's allocation strategy. If females prioritize their own antioxidant protection, an increase in ROS (e.g., induced by increased immune demands) during reproduction might lead to a reduced transfer of antioxidants into the eggs associated with stable or increased antioxidant defenses. However, if females prioritize reproductive investment, they may not alter their antioxidant allocation into the eggs, but show lower antioxidant defenses and suffer higher levels of oxidative damage. Antioxidants are not only important for female's

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antioxidant protection, but also for the offspring development and survival. In fact, it has been shown that carotenoids and other compounds with antioxidant properties (e.g., vitamin E) provide significant protection of embryos and newly hatched chicks against oxidative damage (Surai et al., 1996; Surai and Speake, 1998).

In this study, we tested the hypothesis that a short-term inflammatory process activated by an immunization prior to laying induces oxidative stress in females and, consequently, it influences the deposition of non-enzymatic antioxidants into the eggs. We also hypothesize that a differential antioxidant deposition into the eggs can affect offspring survival. Before egg-laying, female zebra finches (*Taeniopygia guttata*) were injected with lipopolysaccharide (LPS), which mimics a bacterial infection by stimulating the release of cytokines (Akira et al., 2006). LPS induces firstly an inflammatory response by activating the innate immune system (e.g., eosinophils, heterophils and macrophages; Fang et al., 2004; Xie et al., 2000). In birds the acute phase response generally appears within 3 h from the injection of LPS and lasts 24–48 h (Owen-Ashley et al., 2006; Burness et al., 2010; Lopes et al., 2012). Then LPS causes a humoral response with a peak of production of specific antibodies against LPS after 7 days from injection (De Boever et al., 2008; Parmentier et al., 2008). We investigated the effect of the immunization on female's plasma oxidative damage and non-enzymatic antioxidant capacity and the yolk lipophilic non-enzymatic antioxidant capacity. Finally, we assessed the survival of offspring until sexual maturity and whether the egg antioxidant capacity of the second egg of a given clutch predicted the survival of offspring from that clutch. We relied on the non-enzymatic components of the antioxidant defenses because it is the most important barrier against ROS in embryos and hatchlings (Surai and Speake, 1998; Surai, 2002). We predicted that immunization with LPS causes oxidative stress and reduces egg antioxidant capacity because female's antioxidants are depleted due to the immune challenge. We also predicted differences in survival between offspring of immunized mothers and control ones due to the expected differential allocation of antioxidants in the eggs in the experimental groups. Specifically, we predicted that survival probability is lower in the offspring of immunized mothers compared to those of the control group because they should hatch from eggs with lower antioxidant capacity. In statistical terms we expected to find an interaction between egg antioxidant capacity and experimental group.

2. Materials and methods

2.1. Experimental design

Zebra finches used in this study originated from the laboratory colony situated at the Institute of Environmental Sciences of the Jagiellonian University, Cracow, Poland. During the experiments the birds were kept in a climatized room at 20 ± 2 °C, under a 13:11 h incandescent light:dark photoperiod, lights on at 7 am. They were fed ad libitum with a standard mixture of seeds (Megan, Poland), along with a mixture of hard-boiled egg chopped with finely grated carrots. They also received grit and cuttlebone. Vitamins C, A, B1, B6, B12, D3, K (Ornitovit Kanarki, Dolfos, Poland) were added to the food once a week. We randomly paired 64 females with non-related males and we placed the couples into visually separated individual cages (75 × 30 cm and 40 cm high) equipped with external nest-boxes and provided with nest building materials.

Before the experiment the females were allowed to lay one complete clutch in order to gain breeding experience. Once all females had laid the first clutch, we removed their eggs and nests and immunized the females. 34 females were injected intraperitoneally with 100 μl of LPS (Lipopolysaccharide, Sigma Aldrich) suspended in saline (concentration = 1 mg/kg of body weight) and 30 females with 100 μl of saline. A sample of blood was taken from the brachial vein of each female using heparinized capillaries prior to and 24 h after the injection. The capillaries were centrifuged at 2000*g* for 15 min, and then the plasma was separated from the red blood cells and frozen at −70 °C. Because some females did not initiate new clutches after immunization (immunized: 6 out of 34; control: 7 out of 30, $\chi^2 = 0.76$ df = 1 P = 0.38), our final data set consist of 28 immunized and 23 control females.

All eggs were numbered with non-toxic pen just after laying. We removed the second egg from each clutch just after laying and froze it at −70 °C for later analyses of yolk non-enzymatic antioxidant capacity. Given the low variation in the content of single antioxidants among the first eggs in the laying sequence (Griffith et al., 2011; Pariser et al., 2012; Newbrey et al., 2015) or in the yolk antioxidant capacity of the whole clutch (Costantini, 2010; García-Tarrasón et al., 2014), we assumed the antioxidant capacity of the second egg to be representative of that of the whole clutch.

At expected hatching date we inspected the nests hourly during the day, while, during the night (from 8 pm to 8 am), we substituted the eggs with clay models and placed them in an incubator chamber (temperature 36.4 °C, humidity ~70%) to enable determination of which hatchling came from which egg. We returned the unhatched eggs or the chicks hatched during the night in the incubator chamber to the nest in the morning at 8 am.

We marked the nestlings with a non-toxic marker to identify them until they were ringed with an individually numbered aluminum ring at the age of 2 weeks. In order to disentangle pure maternal effects from parental care, half of the nestlings in a given clutch were attempted to be cross-fostered on the day of hatching within a pair of broods (one brood of control and one of immunized female) which started hatching on the same day and had similar clutch size (±1 egg). We matched the nestlings according to the position of the egg in the laying sequence. However, given the small number of nestlings that we were able to cross-fostered, we combined all experimental broods (cross-fostered and untouched) in the statistical analyses (see below). Offspring survival was followed until the third month of life when sexual maturity is achieved and the individual sex can be determined by plumage characteristics. Offspring lived with their parents until adulthood.

2.2. Assessment of female plasma oxidative status

We could not assess oxidative damage compounds and plasma non-enzymatic antioxidant capacity before and after immunization for all females because either the plasma sample was hemolyzed or it was too limited in the amount. Specifically, we could measure the oxidative damage compounds before and after immunization in the plasma of 15 immunized females and 14 control females. We assessed oxidative damage compounds using the d-ROMs test (Diacron International, Italy). This assay quantifies the plasma reactive oxygen metabolites (ROMs), primarily hydroperoxides. In this test the ROMs of the plasma samples, in presence of iron, generate the alkoxyl (R–O•) and alkylperoxyl (R–OO•) radicals that are highly reactive and able to propagate the oxidative cascade. These compounds react with an aromatic amine substituted, contained in the chromogen and oxidized it, changing the color of the mixture to pink. The intensity of the color is directly proportional to the plasma concentration of ROMs (Costantini and Dell'Omo, 2006). We pipetted 4 μl of plasma in a small Eppendorf tube, diluted with 200 μl of a solution containing 0.01 M acetic acid/sodium acetate buffer (pH 4.8) and N,N-diethyl-p-phenylenediamine as chromogen, and incubated for 75 min at 37 °C with mild shaking. Then we centrifuged it at 13,000 rpm for 2 min and pipetted 190 μl of the supernatant into a well of a microplate. We made a calibration curve by 1:1 serial dilution. We read the absorbance at 505 nm with a spectrophotometer (Tecan Infinite M200). Analyses were run in duplicate (repeatability r = 98.87% $F_{72,73} = 88.92$ P < 0.001) and the mean values were used for statistical analyses. The concentration of ROMs was calculated by comparison with a standard curve obtained by measuring the absorbance of a standard solution. The results of the d-ROMs test were expressed as mM of H_2O_2 equivalents.