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Oxidative Stress at Different Stages of the Molting Cycle of Captive *Coturnix coturnix*

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Abstract: Oxidative stress at different stages of the molting cycle of captive, *Coturnix coturnix* was investigated in plasma samples by the determination of the levels of thiobarbituric acid reactive substances and trolox equivalent antioxidant capacity assay by visivel spectrophotometry. An increase of oxidative stress level, a decrease in the total antioxidant status without changes in the levels of thiobarbituric acid reactive substances was observed through molting cycle. There were a negative correlation between the levels of thiobarbituric acid reactive substances and trolox equivalent antioxidant capacity in pre-molt and molt stages.

Key words: *Coturnix coturnix*, oxidative stress, molt, antioxidant, negative correlation, Brasil

INTRODUCTION

The imbalance between the production of pro-oxidative molecules (i.e., Reactive Oxygen Species; ROS) and the efficiency of the antioxidant barriers which comprise a range of endogenous and exogenous compounds antioxidant, leads to oxidative stress in aerobics organisms (Halliwell and Gutteridge, 2007). ROS has a biphasic effects on organisms that is at low dose they modulate cellular communication while at high dose they will damage host tissues and affect the growth, survival and reproduction (Halliwell, 2000). Several studies highlight the ecological and evolutionary roles of oxidative stress in birds (Del Maestro, 1980; Romero, 2004; Costantini, 2008) and give emphasis to the use of animal models under natural and captive conditions to a better understand of these functional interactions among life history traits and place the evolutionary importance of oxidative stress in a more functional perspective (Finkel and Holbrook, 2000; Costantini, 2008; Monaghan *et al*., 2009). Some physiological process of life history of birds are inducers of oxidative stress as the reproduction, immune response, physical activity and molt (Alonso-Alvarez *et al*., 2004; Costantini *et al*., 2007; Costantini and Moller, 2009; Kostelanetz *et al*., 2009). In this context, the molting process can be characterized as a period of considerable physiological and nutritional changes like the increase of the metabolic rate and protein synthesis along with a loss of adipose tissue, bone mass and humoral immune system suppression (Dolnik and Gavrilov, 1979; Brake, 1993; Davis *et al*., 2000; Webster, 2003; Serra *et al*., 2007). These requirements suggested that molt may take place at the expense of the physiological stress response because raising metabolism will increase the production of ROS (Himeno and Tanabe, 1957; Novikov *et al*., 1979; Murphy and King, 1990, 1992; McGraw *et al*., 2003). The levels of oxidative stress biomarkers at different stages of the molting cycle of captive birds are unclear. Earlier reports showed changes in concentrations of corticosterone and suggested that molt is associated with decreased stress responsiveness. The levels of corticosterone serve as a physiological signal to a bird to modify its behavior and metabolism to deal with potentially adverse environmental conditions (Munck *et al*., 1984; Rich and Romero, 2001; Romero, 2004). Therefore, required behavioral adaptations during the molting process could especially in wild birds, drive the observed changes in corticosterone levels. In addition, elevated levels of corticosterone may interfere with protein deposition into feathers (Romero *et al*., 2005, 2006).

The ratio between heterophil lymphocyte is another useful biomarker of oxidative stress in birds and previous reports showed that this ratio was elevated during the molt period (Gross and Siegel, 1986; Maxwell, 1993; Davis *et al*., 2000). Furthermore, the levels of uric acid and glutathione can be potential biomarkers for the evaluation of oxidative stress in birds (Murphy and King, 1990;

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Lin *et al*., 2000; Tsahar *et al*., 2006; Cohen *et al*., 2008a). In this study, we investigate the levels of Thiobarbituric Acid Reactive Substances (TBARS) and Total Antioxidant Status (TAS) at different stages of the molting cycle of captive *Coturnix coturnix*. In addition, the researchrs evaluate the ratio between TBARS and TAS as an index of Oxidative Stress (OS). The researchers expected that the molting cycle increases oxidative stress with the higher level in post molt as a result of an enhanced consumption of antioxidant compounds through molting process.

MATERIALS AND METHODS

Animals and housing: About 30 male of *Coturnix coturnix* were used in the study. Birds were obtained from a commercial hatchery (Belem, Para and BR) at day old. They were weighed and randomly distributed to individual cages (40×45×45 cm) in a room at 25°C and kept on a light: dark cycle similar to the natural cycle (11 L: 13 D in October). Food (seed/pellet mix, grit, vitamins) and water were provided *ad libitum*. Molting is a major event in the annual life cycle of most avian species and may be generally defined as the periodic shedding and replacement of feathers.

In this study, researchers ordered the birds according molt cycle that is pre-molt, molt and post-molt (Murphy and King, 1992; Brake, 1993; Bennett and Owens, 2002). A total of 10 birds were randomly selected at 30th day after starting the experiment and were classified as pre-molt. Subsequently, the feathers were daily examined to determine the start of normal molt event. In this study, 10 birds were selected in a group. The 3rd group was formed by birds for days after the molt event and was considered as pos-molt group. Possession of birds and experimental protocols were approved by Brazilian Institute of Environment (IBAMA).

Experimental design: On the day of experiment, the birds were weighed and the blood sample was collected in heparinized microhemetocrit tubes by puncturing the brachial vein with a 26 gauge needle. Attempt was done to avoid taking >10% of blood volume. Plasma was separated from cells after centrifugation at 2,000 g for 6 min and the samples were immediately analyzed.

Determination of lipid peroxidation (TBARS): Lipid peroxidation was measured by Thiobarbituric Acid Reactive Substances (TBARS) estimation. This method is considered to be a very useful, cheap and easy assay for evaluating oxidative stress (Esterbauer, 1996; Dotan *et al*., 2004; Isaksson *et al*., 2009). Briefly, the lipoproteins are precipitated from the specimen by adding trichloroacetic acid. About 0.05 M and 0.67% Thiobarbituric Acid (TBA) in 2 M sodium sulphate are added to this precipitate and the coupling of lipid peroxide with TBA is carried out by heating in a boiling water bath for 30 min. The resulting chromogen is extracted in n-butanol which is measured at 535 nm. Lipid peroxidation was expressed as nmoles of MDA L^{-1}.

Measurement of Total Antioxidant Status (TAS): The total antioxidant status is a sensitive and reliable marker to detect changes of *in vivo* oxidative stress which may not be detectable through, the measurement of single specific antioxidants (Cohen *et al*., 2007; Cohen and McGraw, 2009).

In this study, total antioxidant status was evaluated by Trolox Equivalent Antioxidant Capacity (TEAC) assay. This method is based on the suppression of the absorbance measured at 740 nm of radical cations of 2, 2-azinobis (3-ethylbenzothiazoline, 6-sulfonate) (ABTS) by antioxidants presents in the sample in a degree which is proportional to their concentration. The antioxidant capacity of samples was expressed as Trolox Equivalent Antioxidant Capacity (TEAC) by using the calibration curve plotted against different amounts of Trolox (Prior and Cao, 1999; Re *et al*., 1999).

Oxidative Stress level (OS): Oxidative stress was considered as the ratio between TBARS and TAS (Yeum *et al*., 2004; Cohen and McGraw, 2009). This ratio is affected by the anti-oxidant response of the organism against pro-oxidant production with higher values meaning higher oxidative stress. Researhers used this ratio because these variables are significantly correlated (Esterbauer, 1996; Halliwell and Gutteridge, 2007; Costantini and Verhulst, 2009).

Statistical evaluation: Data are reported as means±SD. Data sets were tested for normality by Kolmogorov Smirnov. Pearson coefficient was used to determine the correlation between the concentrations of TBARS and TAS in the plasma.

The comparison between the TBARS and the TAS levels at different stages of the molting cycle was performed by ANOVA with random term block and fixed term treatment. When general linear model yielded significant differences, Duncan's multiple range test was conducted to determine particular differences between treatments.

Statistical analyses were performed with STATISTICA software package (Version 6, Stat Soft 2001, Tulsa, USA). Significance was accepted at $p<0.05$.

RESULTS AND DISCUSSION

The variables at different stages of the molting cycle were normally distributed. TBARS and TAS concentrations and OS ratio are shown in Table 1. There were no statistically significant differences in mean concentrations of TBARS through molting cycle ($F = 0.72$, $p = 0.502$). The mean plasma concentration of TAS differed significantly through molting process; the highest concentration was measured before molting and the lowest in post-molt ($F = 5.2$, $p = 0.014$). The plasma concentration of TAS in post-molt was significantly lower than the others phases while that of the pre-molt and molt did not differ one from the other.

Plasma concentrations of TBARS and TAS were negatively correlated in pre-molt and molt (Pre-molt: $r = -0.7494$, $p = 0.05$; Molt: $r = -0.3434$, $p = 0.33$) but positively correlated in post-molt ($r = -0.04$, $p = 0.957$). OS level differed significantly through molting process; the highest ratio was determined in post-molt ($F = 4.71$, $p = 0.0205$) while that of the pre-molt and molt did not differ one from the other.

The study showed an increase of OS with a decrease in TAS without changes in TBARS levels through molting process of captive *Coturnix coturnix*. This feature reflect the changes in correlations of these variables through molt cycle and may be related to an enhanced production of ROS and the subsequent response of enzymatic and non enzymatic antioxidants defenses which sustained the low levels of ROS through molting process as well as to the reduction in the resources of non enzymatic antioxidants and consequently reallocation of essential nutrients as vitamins, minerals, essential amino and fatty acids, lipotropic factors and carbohydrates (Brake and McDaniel, 1981; Alonso-Alvarez *et al*., 2006; Catoni *et al*., 2008; Costantini, 2008; Cohen *et al*., 2008a, b; Strochlic and Romero, 2008; Costantini and Verhulst, 2009).

Antioxidant systems vary greatly among tissues, species, phase of the life cycle or on the age of an individual and this heterogeneity could mirror different response-related oxidative stress and consequently life histories, intensities of sexual selection, histories of parasite-mediated selection and/or feeding habits (Lozano, 1994; Surai *et al*., 1996; Alonso-Alvarez *et al*., 2007; Costantini *et al*., 2007; Cohen *et al*., 2008a; Catoni *et al*., 2008; Cohen and McGraw, 2009; Isaksson *et al*., 2009). In many tissues and primarily in mitochondria, antioxidant enzymes are the key defense against oxidative damage. In circulating systems, micro molecular antioxidants such as uric acid, vitamins C and E and carotenoids play a more important role. Antioxidant enzymes which have a specialized function are likely regulated to adjust antioxidant protection specifically whereas, micro molecular antioxidant levels depend on dietary intake and on their other physiological roles such as signaling (Surai *et al*., 1996; Finkel and Holbrook, 2000; Alonso-Alvarez *et al*., 2006; Halliwell and Gutteridge, 2007; Catoni *et al*., 2008; Cohen *et al*., 2008a, b).

Table 1: Plasma levels of Thiobarbituric Acid Reactive Substances (TBARS), Trolox equivalent antioxidant capacity assay (TAS) and Oxidative Stress level (OS) at different stages of the molting cycle of captive *Coturnix coturnix*

Molt phases	N	TBARS	TAS	OS
Pre-molt	10	1.5±0.5	0.6±0.1	2.7±1.8
Molt	10	2.1±1.0	0.4±0.1	4.8±3.1
Post-molt	10	1.9±0.8	0.3±0.1	7.7±3.3

TBARSs are expressed as mM L^{-1}, TAS as Trolox equivalent Antioxidant capacity, OS is the ratio between TBARS and TAS; results are expressed as mean = SD

The researchers confirm, the hypothesis that oxidative stress levels enhance thought the molt cycle and suggested that the risk of harmful effects of ROS was enhanced in the post molt. However, the balance in physiological systems is complex, especially in birds in which molecular mechanisms allowing them to defend against free radicals are based on their low rate of mitochondrial oxygen radical production and a better molecular protection against pro-oxidant molecules (Barja, 1998; Finkel and Holbrook, 2000; Costantini *et al*., 2007; Monaghan *et al*., 2009). Thus, studies to better understand how oxidative stress in post-molt influences the eggs quality and represent a trait of natural history of wild birds should be encouraged.

CONCLUSION

There were a negative correlation between the levels of thiobarbituric acid reactive substances and trolox equivalent antioxidant capacity in pre-molt and molt stages.

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