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Institute of Genetics and Animal Breeding, Jastrzębiec, Poland

Activity of antioxidant enzymes and levels of GSH and MDA in heritage breed and commercial crosses hen eggs

Renata Muchacka[1]*, Anna Charuta[2], Ewa Sosnówka-Czajka[3], Iwona Skomorucha[3], Joanna Marchewka[4], Edyta Kapusta[1]

[1] Department of Animal Physiology and Toxicology, Institute of Biology, Pedagogical University of Cracow, Podbrzezie 3, 30-054 Cracow, Poland

[2] Department of Emergency Medicine, Faculty of Natural Science, Siedlce University of Natural Sciences and Humanities, Konarskiego 2, 08-110 Siedlce, Poland

[3] Department of Technology, Ecology and Economics of Animal Production, National Research Institute of Animal Production, Krakowska 1, 32-083 Balice n. Cracow, Poland

[4] Department of Animal Behaviour, Institute of Genetics and Animal Breeding Polish Academy of Sciences, Postępu 36A, 05-552 Magdalenka, Poland

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The objective of this study was to determine the activity of antioxidant enzymes and the level of GSH and MDA in yolk and albumen of eggs laid by 48 and 50 week old hens. In the experiment 180 hens of three heritage breeds (Yellowleg Partridge, Sussex, Leghorn) and 180 commercial crosses of laying hens (Hy-Line, ISA Brown, Lohmann) were used. Layers were reared on litter and had no outdoor access. Birds were assigned to treatment groups I to VI (Yellowleg Partridge, Sussex, Leghorn, and commercial layers Hy-Line, ISA Brown and Lohmann, respectively). Birds were fed standard diets based on concentrates for laying hens *ad libitum* and had free access to water throughout the experiment. All the groups were managed under uniform environmental (air humidity and temperature, lighting programme) and feeding conditions. At 48 and 50 weeks of age, 6 eggs from each group were collected. In the samples of egg yolks and albumens the concentration of GSH and MDA, and activity of SOD, GPx and CAT were estimated. The egg yolks and egg albumens showed statistically significant differences in SOD activity, GSH and MDA levels in both studied periods. At 48 and 50 weeks of age, the highest activity of SOD and the lowest level of MDA and GSH were observed in eggs from Sussex hens (heritage breeds) and in eggs from Hy-Line

*Corresponding author: renata.muchacka@up.krakow.pl

(commercial crosses). In conclusion, it can be stated that the genetic background of laying hens influences the activity of antioxidant enzymes and levels of GSH and MDA in eggs, which may influence their quality. The differences between the treatment groups in the measurements were maintained in both studied periods.

KEYWORDS: antioxidant enzymes / egg / GSH / MDA / origin of hens

Eggs have high nutritional value in human diet due to their favourable chemical composition, since an egg contains all the ingredients needed for the development of the embryo. Egg protein is a colloidal solution of proteinaceous substances in aqueous mineral salts and carbohydrates, while yolk is a colloidal solution of proteinaceous substances and carbohydrates in aqueous mineral salts containing fat suspended in the form of emulsions. Eggs are important for the human and animals since their components can be utilized to build and renew body tissues [Seuss-Baum 2007]. The chemical composition of eggs is somewhat volatile and depends on multiple factors (environment, laying period, the age of the hens, feed) [Pingel and Jeroch 1997, Jiang and Mine 2000, Skrivan *et al.* 2005, Seuss-Baum 2007, Krawczyk 2009]. The quality and durability of eggs and other food products deteriorates with the storage time [Okolie *et al.* 2009]. Spoilage of food is caused by complicated chemical processes, but most of the changes are caused by the presence of reactive oxygen species (ROS) [Choe and Min 2005]. Both free radicals and antioxidants occur naturally in the body, because they are necessary for many life conditioning processes. As long as there is a balance between free radicals and antioxidants, the body is functioning properly. However, a so-called oxidative stress is involved when it comes to increased production of free radicals or decreased activity of antioxidants [Betteridge 2000, Jóźwik *et al.* 2010, 2012, Dasuri *et al.* 2013]. ROS can damage all the biomolecules which are present in the body - including protein, fat, and DNA, leading to mutations. In extreme cases, oxidizing agents can lead to cell death [Gutteridge 1995, Liochev 2013]. Antioxidants prevent the destructive effects of free radicals, giving them their free electron, and thus remove their excess from the body [Irshad and Chaudhuri 2002]. Egg, as a diet component may play a role in the development of oxidative stress in its consumers organism.

The aim of the study was to determine the differences in antioxidant enzymes activity and differences in the levels of reduced glutathione and malondialdehyde in eggs from heritage breeds and commercial crosses of hens at 48 and 50 weeks of age.

Material and methods

Hens and experimental design

The experiment was carried out using 180 hens of three heritage breeds (Yellowleg Partridge, Sussex, Leghorn) and 180 commercial crosses of laying hens (Hy-Line, ISA Brown, Lohmann). Birds at the age of 18 weeks were assigned to groups I to VI (Yellowleg Partridge, Sussex, Leghorn, and commercial layers Hy-Line, ISA Brown

and Lohmann, respectively) and were kept up to 56 weeks of age. Each group was subdivided into 3 subgroups, each consisting of 20 birds. Layers were reared in the litter system at a stocking density of 9 birds per m^2 and had no outdoor access. Birds were fed standard diets based on concentrates for laying hens *ad libitum*. Hens were fed the same basal diet containing 30% wheat, 26% maize and 14% extracted soybean meal (CP 17%, ME 11.4 MJ · kg^{-1}, Lys 0.80%, Met 0.38%, retinyl acetate 10000 IU, tocopherol 25 mg, vitamin D 50 mg, Ca 35.3 g, and P (available) 3.4 per kg). Birds had free access to water throughout the experiment. All the groups were managed under uniform environmental (air humidity and temperature, lighting programme) and feeding conditions. A lighting schedule of 16 h of light and 8 h of darkness (16L:8D) was applied.

Sample collection and laboratory analyses

In total, the 36 eggs (6 eggs from each group) were collected from layers at 48 and 50 weeks of age. Eggs were evaluated after 24 h of refrigerated storage at 4°C and 55% humidity. In the samples of egg yolks and albumens the activity of superoxide dismutase (SOD), glutathione peroxidase (GPx) and catalase (CAT) and concentration of reduced glutathione (GSH) and malondialdehyde (MDA) were evaluated.

Superoxide dismutase (SOD) activity. The yolks and albumens samples were homogenised in 5 ml of 20 mM HEPES buffer with pH 7.2, chilled to 4°C (1 mM EDTA (PubChem CID:8759), 210 mM mannitol (PubChem CID:6251), and 70 mM sucrose (PubChem CID:5988) per 1g of tissue). The homogenates were centrifuged at the speed of 1.500 x g for 5 min at a temperature of 4°C. The SOD analysis, conducted using the Cayman Chemical Company test (Ann Arbor, Michigan 48108 USA), enabled collecting data of all three types of SOD (Cu/Zn, Mn, FeSOD). The reactions occurring during the test were based on the use of a tetrazolium salt to detect reactive oxygen species generated by xanthine and hypoxanthine. Absorbance was read at three wavelengths ($\lambda_{440, 450, 460}$) using a Synergy4 microplate reader (Biotek; Winooski, Vermont 05404 USA). Calculations of SOD activity were made with Gen5 software (Biotek). The activity of SOD was expressed in U/mg of protein.

Catalase (CAT) activity. All the samples were incubated at 37°C for 20 minutes, and then centrifuged with the subsequent supernatant diluted at 1:2 ratio with sample buffer. Shielded from light, 20 µl of each sample was pipetted into a 96-well plate in duplicate and then incubated on a shaker at room temperature for 20 minutes with 100 µl assay buffer, 30 µl methanol and 20 µl hydrogen peroxide. Then 30 µl KOH was added to terminate the reaction and the wells were incubated with 30 µl purpald for another 10 minutes at room temperature. The final incubation with 10 µl KIO_4 lasted for five minutes, after which the plate was immediately placed in the plate reader and analyzed against the formaldehyde standard at 540 nm. The activity of CAT was expressed in U/mg of protein.

Glutathione peroxidase (GPx) activity. The samples were homogenized in 5 mL of the buffer, chilled to 4°C, consisting of 50 mM Tris-HCL, 5 mM EDTA and 1 mM dithiothreitol and were centrifuged at 10,000 x g for 15 min at 4°C. The obtained supernatant was stored on ice until the analysis. The activity of GPx was determined

using the Cayman Chemical Company test (Ann Arbor, Michigan 48108 USA). The oxidation reaction of NADPH to $NADP^+$ enabled detecting changes in absorbance ($\lambda 3_{40}$). Absorbance was read and measurements of reaction kinetics were performed using a microplate reader Synergy4 (Biotek; Winooski, Vermont 05404 USA). The results were calculated using the Gen5 software (Biotek). The activity of GPx was expressed in U/mg of protein.

Malondialdehyde level. The yolks and albumens samples were homogenised in 2 mL of a phosphate buffer, chilled to 4°C, with the addition of 20 µL of butylated hydroxytoluene in acetonitrile. The samples were centrifuged at 10,000 x g for 10 min at the temperature of 4°C. The obtained supernatant was stored on ice until the analysis. The further procedure strictly followed instructions of the producer of the OxisReasearch™ Bioxytech® MDA-586™ test (Foster City, California 94404-1136 USA). The absorbance ($\lambda 5_{86}$) was read using the Cary Varian 50Bio spectrometer (Santa Clara, California 95051 USA). Calculations were performed on the basis of a calibration curve obtained according to the producer's recommendations and the template included in the test report. The MDA level was expressed in nM/mg of protein.

Glutathione, Glutathione disulphide content. Glutathione concentration was determined in the yolks and albumens by means of the OxisReasearch™ Bioxytech® GSH/GSSG - 412™ test (Foster City, California 94404-1136 USA). Before the analysis, the samples were frozen with the addition of M2VP (1-methyl-2-vinyl-pyridium trifluoromethane sulphonate) at a temperature of -80°C. The released reduced glutathione (GSH) concentration levels were determined in accordance with the detailed instruction provided by the kit's producer. Absorbance reading (λ_{412}) and the measurement of reaction kinetics were performed using a microplate reader Synergy4 (Biotek; Winooski, Vermont 05404 USA). The results were calculated using Gen5 software (Biotek). Glutathione concentration was expressed in µM/g of protein.

Statistical analysis

All data were analysed by analysis of variance procedures (ANOVA) and Duncan's multiple-range test using computer program Statistica version 10.0 PL. The data were expressed as means ±SEM and were considered as significant when P values were less than 0.05.

Results and discussion

Hen eggs are a very important food products for human consumers. They can be ingested unprocessed, processed, or as ingredients of other food products [Raikos *et al.* 2006]. ROS processes occurring in food, also in eggs, lead to a reduction in its durability and quality [Okolie *et al.* 2009].

There are many factors influencing the egg internal and shell quality, including the origin of laying hens, nutrition, veterinary prevention and zootechnical supervision of birds. The quality of eggs depends also on the hen's age, productivity and farming

system [Pingel and Jeroch 1997, Roberts 2004, Van den Brand *et al.* 2004, Basmacioglu and Ergul 2005].

The formation of free radicals, including reactive oxygen species (ROS), is largely related to the natural oxidative-reduction processes (respiratory chain changes) occurring in the mitochondria of each cell [Dröge 2002]. Endogenous sources of reactive oxidant forms include primarily mitochondria (respiratory chain), macrophages, neutrophils, peroxisomes, and some enzymes such as NADPH oxidase, xanthine oxidase, NO synthase (NOS), cyclooxygenase, lipoxygenase [Betteridge 2000, Dasuri *et al.* 2013]. In addition, ROS act as signaling molecules (second type relays) in a process called redox signaling. They activate the internal defenses and repair of the body, as well as the antioxidant defense system [Juranek *et al.* 2013, Liochev 2013, Murphy *et al.* 2011].

Although free radicals have a positive effect on many processes occurring in the body, such as cell growth regulation, hormone secretion, muscle contraction, and intracellular signaling [Dröge 2002, Śliwa-Jóźwik *et al.* 2002], their excess has strong cytotoxic effects [Dasuri *et al.* 2013, Hekimi *et al.* 2011]. When the concentration of free radicals grows, they become dangerous because of the damage which they cause [Hekimi *et al.* 2011].

To prevent the destruction caused by uncontrolled reactions of ROS, living cells produce antioxidant protection [Irshad and Chaudhuri 2002, Yu 1994]. Capture and elimination of ROS is the responsibility of enzymatic and non-enzymatic antioxidant systems. The natural enzyme system for the removal of free radicals includes superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx) [Sies 2013, Lipińska *et al.* 2017]. Their effective function depends on the content of dietary trace elements such as selenium and manganese [Horbańczuk and Wierzbicka 2017, Pogorzelska *et al.* 2018, Sarmadi and Ismail 2010]. Generally, egg yolk shows a higher ability to accumulate selenium than does the albumen. The antioxidant effect of selenium administered in hen feeds is the increased activity of selenoenzyme, i.e. glutathione peroxidase in egg yolks. This is equivalent to the improvement of their oxidative stability during storage [Gutierrez *et al.* 1997]. In our study, egg yolks and egg albumens (Fig. 1-3) from Sussex (heritage breed) and Hy-Line (commercial cross) hens showed the highest activity of antioxidant enzymes at 48 and 50 weeks of age, but statistically significant differences were observed only in the case of SOD activity (Fig. 1). The activity of SOD in egg yolks and albumens from Sussex hens was significantly higher ($p<0.01$, weeks 48 and 50) compared to Yellowleg Partridge and Leghorn hens. The highest activity of SOD in egg yolks and albumens from commercial crosses of hens was observed in Hy-Line vs. ISA Brown ($p\leq0.05$, weeks 48 and 50) and Lohmann ($p\leq0.01$, weeks 48 and 50). A similar trend has been observed in GPx activity in egg yolks and albumens, but there were no statistically significant differences (Fig. 2).

There were no significant differences in CAT activity in the examined eggs (Fig. 3). However, it is worth noting that the activity of CAT in eggs from hens of heritage

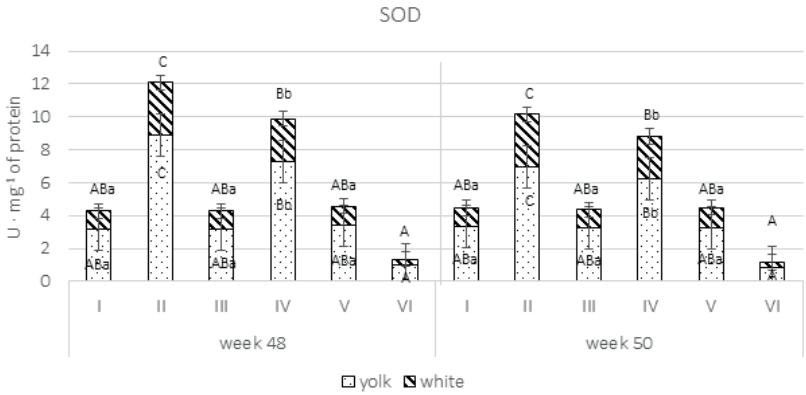

Fig. 1. Superoxide dismutase activity in egg yolks and albumens from laying hens aged at the week 48 and 50 weeks. a, b – values with different letters differ significantly at P≤0.05; A, B – values with different letters differ highly significantly at P≤0.01.

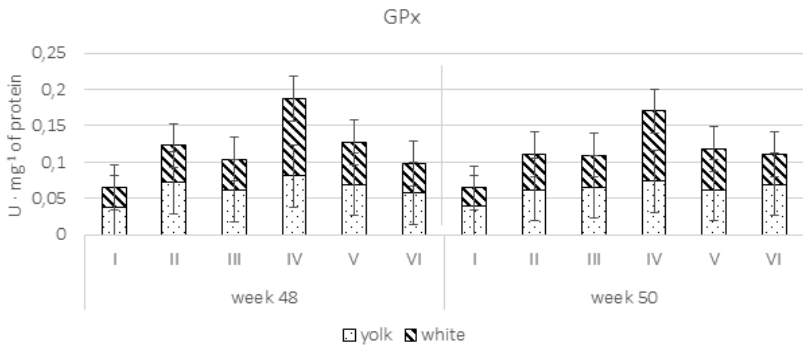

Fig. 2. Glutathione peroxidase activity in egg yolks and albumens from laying hens at the week 48 and 50.

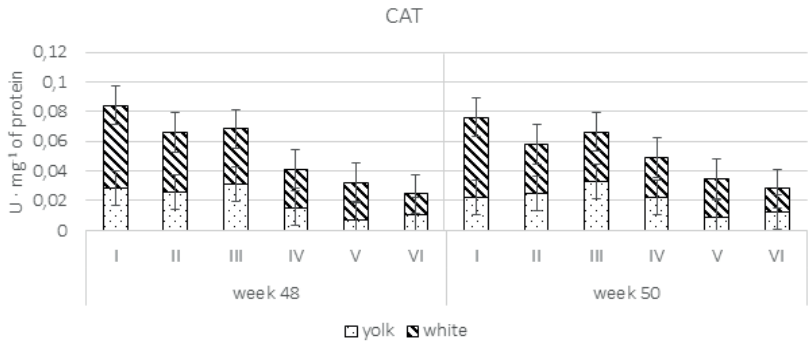

Fig. 3. Catalase activity in egg yolks and albumens from laying hens at the week 48 and 50.

breeds was higher (p>0.05, weeks 48 and 50) than in eggs from commercial crosses of hens.

Glutathione, selenium, vitamin C and E are the main non-enzymatic antioxidants [Sarmadi and Ismail 2010, Sies 2013, Jasińska and Kurek 2017]. The highest concentration of GSH at 48 and 50 weeks of age was characteristic for egg yolks and albumens from Leghorn hens (among the heritage breeds) and from ISA Brown hens (among the commercial crosses of hens) (Fig. 4).

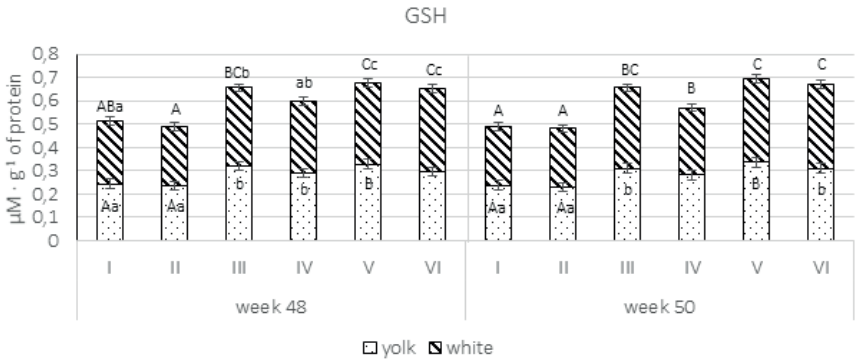

Fig. 4. Glutathione level in egg yolks and albumens from laying hens at the week 48 and 50. a, b – values with different letters differ significantly (P≤0.05); A, B – values with different letters differ highly significantly (P≤0.01).

Significant differences in GSH levels were recorded in egg yolks from Leghorn compared to Yellowleg Partridge (p≤0.05, weeks 48 and 50) and Sussex hens (p≤0.05, weeks 48 and 50), and in egg albumens from Leghorn compared to Yellowleg Partridge (p≤0.05 at week 48, P≤0.01 at week 50) and Sussex hens (p≤0.01, weeks 48 and 50). Among the commercial crosses of hens, statistically significant differences in GSH levels were found only in albumens of eggs collected at the 48th (p≤0.05) and 50th (p≤0.01) week of rearing.

One of the effects of ROS is lipid peroxidation. Malondialdehyde (MDA) is formed during oxidation of polyunsaturated fatty acids. MDA is formed by the action of free radicals inside the body [Long *et al.* 2009], as well as in animal products containing fats [Okafor *et al.* 2007, Okolie *et al*. 2009, Marzoni *et al.* 2014]. This compound is also the most commonly used indicator of the intensity of oxidation of fats associated with the increased generation of reactive oxygen species. In our study, the lowest level of MDA was observed in egg yolks and albumens of Sussex hens and the highest in the eggs of Leghorn hens (p≤0.05 at week 48). Among the commercial crosses of hens the highest levels of MDA in the albumens (p≤0.05, week 48 and 50) and yolks (p≤0.05 at week 48, p≤0.01 at week 50) were observed in Hy-Line hens, and the lowest in Lohmann hens (Fig. 5).

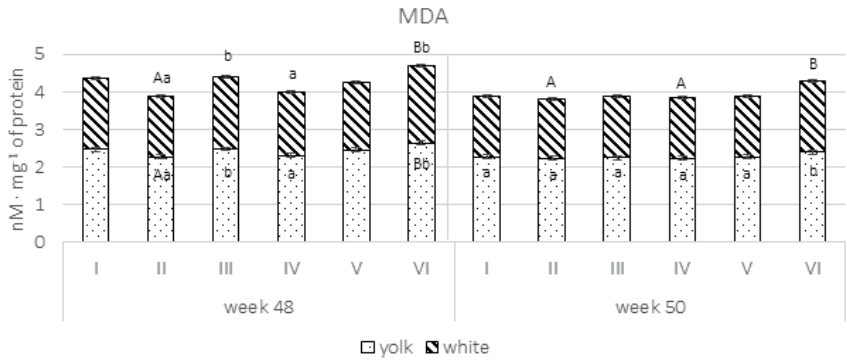

Fig. 5. Malondialdehyde level in egg yolks and albumens from laying hens. a, b – values with different letters differ significantly at P≤0.05; A, B – values with different letters differ highly significantly at P≤0.01.

It is important from the point of view of food safety that cold storage or freezing of raw material only slightly inhibits the peroxidation process [Okolie *et al.* 2009, Horbańczuk and Wierzbicka 2016]. This is because the solubility of free lipid radicals in the lipid fraction increases at low temperatures, so that they diffuse over long distances, inducing lipid oxidation reactions during storage [Kanner 1994].

Activities of antioxidant enzymes and levels of GSH and MDA in the egg yolks and albumens indicated that they vary according to the breed/origin of hens. Among the heritage breeds, the highest quality in terms of measurements was shown by Sussex hens and among the commercial crosses of hens – by Hy-Line. The differences between the groups in the measurements were consistent with the aging of the hens.

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