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BIOMARKERS OF OXIDATIVE STRESS: POSSIBLE PROSPECTIVE AND APPLICATION IN ECOTOXICOLOGY AND HEALTH MONITORING IN AVIAN SPECIES

Coordinatore:

Chiar.mo Prof. Donofrio Gaetano

Tutore:

Chiar.mo Prof. Bertini Simone

Dottorando: Iemmi Tiziano

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INTRODUCTION

Cap. 1 - Free radicals and Oxidative Stress: general framework

1.1 - Free radicals and oxidative stress

Free radicals are atoms, ions, or molecules characterized by the presence of an unpaired electron on the outermost orbital. This determines an instability due to the tendency to equalize the number of electrons, leading these compounds to bind with other radicals or to steal an electron from other molecules by altering their structure, function and converting them into free radicals in a chain reaction. Carbohydrates, lipids, proteins, and nucleic acids are molecules subject to aggression by free radicals, which leads to denaturation of the macromolecules and consequent loss of biological functionality. Whenever this phenomenon occurs to cell components, physiological cellular functions are compromised, possibly leading to apoptosis or necrosis (Muller *et al.* 2007).

The formation of free radicals can occur by homolysis (as opposed to heterolysis which involves the formation of ions), by chain reaction with another radical, or by redox reaction (Cheeseman *&* Slater 1993). In homolysis radicals are formed following the breaking of a covalent bond (i.e. $Cl_2 \rightarrow \cdot Cl + \cdot Cl$). The homolytic rupture of the covalent bond generally requires high energy, that may derive from various sources such as pyrolysis, from the investment by the flow of electrons (voltaic arc), or exposure to high-energy waves (electromagnetic or corpuscular), the consequent breaking of the bond involves a great energy dispersion itself, resulting in an exothermic manifestation (Solomons *&* Fryhle 2000).

In the chain reaction, a free radical steals an electron from a compound resulting in the formation of a new radical. The formation of radicals following a redox reaction occurs because of a redox reaction itself or as a consequence of reactions under electrolysis conditions (Solomons *&* Fryhle 2000).

Free radicals are divided into "short-lived" radicals, highly unstable and in continuous interaction with the surrounding matter, and "long-lived" radicals, , in turn, can be classified into three categories, stable radicals, persistent radicals and thinning radicals, these last compounds characterized by a double radical nucleus (Linde *et al*. 1999).

An example of a stable radical is molecular oxygen O_2, which despite the even number of electrons (e^-) has paramagnetic properties deriving from the distribution of electrons in molecular orbitals, or radicals deriving from antioxidant molecules such as α-tocopherol, a compound of the family of vitamins of group E, these by their nature tend to be not very reactive with limited interference with the surrounding molecules (Linde *et al.* 1999).

On the contrary, persistent radicals are organic molecules characterized by an important stearic encumbrance surrounding the radical center, which limits its interaction with matter, nevertheless, maintaining an important reactivity. These are compounds that are abundantly released after combustion, being at the basis of the oxidative and carcinogenic activity of fumes and combustion residues (Lomnicki *et al*. 2008).

In general terms, free radicals can originate because of (Yoshikawa & Naito 2002; Lomnicki *et al.* 2008):

- Exposure to ionizing radiation (UV-ray, X-ray, or γ-radiation)
- From combustion, which is a highly exothermic radical chain reaction
- Exposure to physical events, such as hydrolysis and plasma arc formation
- At a biological level, during inflammatory processes, in which the leukocytes through enzymatic processes (i.e. activation of NADH oxidase) produce Reactive Oxygen Species (ROS) intended for the aggression of elements recognized as non-self
- At the level of excretory tissues following enzymatic degradation processes of certain xenobiotic compounds (i.e. carbon tetrachloride)
- Cellular enzymatic processes related to apoptotic phenomena
- Cellular respiration processes in aerobic organisms, firstly at the mitochondrial level, consequently to oxidative phosphorylation
- Exposure to transition metals, with catalytic activity in the formation of free radicals
- The interaction of organic molecules with other free radicals of exogenous origin, including molecular oxygen.

All living organisms are continuously exposed to the harmful effects of free radicals. ROS are the main harmful agents in the aerobic environment: they are generated by endogenous processes (. e. cellular respiration, metabolic reactions, or immune response) or of exogenous origin (i.e. food-derived peroxides, chemicals, radiations). In physiological conditions, the degenerative action on cellular components is kept under control by the presence of an efficient antioxidant system, made of both endogenous (i.e. glutathione/glutathione peroxidase complex, superoxide dismutase, and

catalase) and exogenous (mostly of nutritional origins, such as tocopherol and ascorbic acid) antioxidants (Yoshikawa *&* Naito 2002). However, when the oxidative pressure overcomes the protective ability of the antioxidant system, an Oxidative Stress (OS) takes place (Birden *et al.* 2012).

1.1.a - *Endogenous sources of free radicals*

The use of O_2 in the metabolic energy-generating process at mitochondrial levels in the aerobic metabolism creates reactive oxygen metabolites (ROMs). Among ROMs, are the most important are the superoxide anion (O_2^-), hydrogen peroxide (H_2O_2), hypochlorous acid (HClO) and the hydroxyl radical ($\cdot OH^-$) (Raha *&* Robinson 2000). Again, following to leucocyte activation, ROMs can be produced and released in the phagolysosome, to degrade phagocytosed pathogens (Birden *et al.* 2012).

After being produced, ROMs can exit from the origin organelles and diffuse into the cytoplasm, into the cell nucleus and the intercellular compartment. In these districts ROMs can undergo spontaneous chemical variations following the interaction with the present molecules, including antioxidants, or they can be intercepted by enzymatic complexes.

An important role in the formation and realise of H_2O_2 is played by the humoral cells of the white line, in particular by the action of the eosinophilic peroxidase and of the myeloperoxidase where the H_2O_2 intervenes both as a transmitting molecule and as an oxidizing compound in contrast to antigenic highlighting (Birden *et al.* 2012). In addition to the direct oxidizing action, following the interaction of H_2O_2 with Cl^- ions, it incurs the formation of HClO, highly reactive ROS and one of the main reactants involved in cell damage from radicals (Klebanoff 2005).

The peroxide then by Haber-Weiss Fentom reaction, catalysed by metal ions such as Fe^{2+} and Cu^+, determines the formation of $\cdot OH^-$, the most reactive among the ROS able to easily attack organic molecules. Furthermore, other important ROS are the peroxide radical ($ROO\cdot$) and the hydroperoxide radical ($HOO\cdot$), involved in the phenomena of lipid peroxidation (Birden *et al*. 2012).

The biological activity therefore leads to a natural production of oxidizing products. These activities can be implemented, and therefore the synthesis of ROS, in case of increment in the metabolic activity of the organism, for example as a result of reproductive activity, the immune response and physical activity (Costantini 2008).

In avian species, the weight of the reproductive activity on the parents is experimentally verifiable through the variation in the number of eggs and therefore pullets reared per litter. For example, in the kestrel (*Falco tinnunculus*) the artificial increase in the number of eggs, and consequently in the number of offspring, produced a reduction in the life expectancy of parents: this is an effect likely

related to an increase in the levels of OS associated with overmanage (Daan *et al.* 1996; Costantini 2008).

In immune activation leukocytes release free radicals and pro-oxidant factors. The onset or not of OS, and the consequent overcoming of the physiological antioxidant capacities, depends on the extent of the immune stimulation, in correlation to the physiological state of the subjects (Costantini 2008).

Physical overexertion also determines an increase in OS, on the contrary, physical activity within physiological limits does not cause detectable stress: for example, a reduction in plasma antioxidant capacity and an increase in plasma free radicals have been observed in travelling pigeons subjected to long distances (Costantini 2011).

1.1.b - *Exogenous sources of free radicals*

An important source of free radicals and oxidants are combustion and pyrolysis processes, for example resulting from cigarette smoke and fumes from internal combustion engines; the primary route of intake is respiratory, but it is also possible *per os* intake through the ingestion of carbonization products (Birden *et al.* 2012). The radical activity may depend both on the presence of radical compounds and on the activation of phlogogenic phenomena, due to the presence of corpuscular material, particularly if inhalated (Birden *et al*. 2012).

Exposure to ozone (O_3) can cause lipid peroxidation and leukocyte activation with the release of phlogogenic factors with a consequent increase in oxidative phenomena (Bouthillier *et al*. 1998).

Ionizing radiations in the presence of O_2 determines the conversion of hydroxyl radicals and the superoxide group into hydrogen peroxide or associated with radicals of organic macromolecules. The peroxides in turn, in the presence of metal ions by Fentom reaction, are reduced into hydroxyl radicals which will oxidize surrounding compounds (Biaglow *et al.* 1992).

Heavy metals can increase oxidative metabolism in two ways: firstly by catalysation through the Fentom reaction, inducing the peroxidation of lipids in the presence of O_2; in second place interfering with the functionality of the enzymes with antioxidant action, preventing their function scavenging(this is the case for example of As, Zn and Pb) (Reid *et al.* 1994).

Another way of introducing ROS is through the intake of altered foods rich in peroxides and free radicals (i.e. rancid fats) or through the intake of certain xenobiotics, including most of the pre-carcinogenic molecules that, as a result of phase 1 reactions (oxidation, hydrolysis and reduction) primarily in the liver promoted by cytochrome P450, acquire radical activity (Pryor 1982).

1.2 - Antioxidant mechanisms

As we have seen, organisms, and in particular aerobic organisms, are continuously exposed to oxidants and free radicals of exogenous and endogenous origin. To compensate for the constant oxidative aggression, they have developed antioxidant mechanisms, consisting of a system of enzymes and acting substances antioxidant (Birben *et al.* 2012). Characteristic of antioxidants is that they are particularly subject to oxidation, diverting the action of radicals from the surrounding compounds (Wolf 2005).

The antioxidant action carried out by these molecules is realized by intervening as reducing agents, therefore as electron acceptors, with the consequent transformation of the molecule itself into a radical but characterized by high stability and therefore not inclined to release the acquired electron, consequently, to block the chain reaction (Rice-Evans *et al.* 1995). The antioxidant molecules, such as α-tocopherol or glutathione, can then be brought back into the reduced form to return to carry out their antioxidant action again. Given the low propensity to release electrons normally at a biological level, there are enzymatic systems capable of to lower the energy necessary for the removal of the electron, transferring it to accepting molecules. Therefore, the most efficient antioxidant mechanisms provide for the synergistic intervention of several compounds, where the action of one component depends on the presence and correct functionality of the others. The protective efficiency provided by an antioxidant depends on its concentration, its reactivity towards the particular ROS considered, and the state of the antioxidants and enzymatic complexes with which it interacts (Vertuani *et al.* 2004). The antioxidant molecules can be grouped into two categories: hydrophilic and lipophilic.Generally, hydrophilic compounds appear to be abundant in cell cytoplasm and plasma, while lipophilic compounds in cell membranes (Sies 1997).

1.2.a – <u>Food-borne antioxidant molecules</u>

α-tocopherol is the most assimilable and present form at animal level of the complex of molecules identifiable with the generic term Vitamin E. They are lipophilic compounds, phenolic molecules of food origin, the main and most abundant antioxidant to protect membrane integrity, with a contrasting action particularly against peroxides (Scott 1997).

Vitamins of E group intervene by donating hydrogen from the hydroxyl group (-$OH^{\cdot}$) on the ring structure to free radicals, inactivating them. By donating the hydrogen, the phenolic compound itself becomes a relatively non-reactive free radical, because the unpaired electron on the oxygen atom is usually delocalized into the structure of the aromatic ring, thus increasing its stability (Scott

1997). The main biological role of vitamin E is to preserve from oxidation by free radicals the Poly Unsaturated Fatty Acids (PUFAs) constituting the membrane phospholipids, other lipophilic components of cell membranes, and low-density lipoproteins (LDL). Vitamin E is found mainly within the phospholipid bilayer of cell membranes. It is particularly effective in preventing lipid peroxidation, a series of chemical reactions that result in the oxidative deterioration of PUFAs. Elevated levels of lipid peroxidation products are associated with numerous diseases and pathological conditions (Duthie 1993). Although vitamin E is found mainly in cell membranes and organelles where it can exert its maximum protective effect, its concentration can be at most one molecule per 2000 phospholipid molecules. This suggests that after its reaction with free radicals it is rapidly regenerated, possibly by other antioxidants, firstly ascorbic acid, but also retinol and ubiquinone, which act as an electron acceptor, bringing the vitamin E molecules back into reduced shape. (Kagan 1998; Wang *&* Quinn 1999).

The primary oxidation product of α-tocopherol is a tocopheryl quinone, the compound, if not reported in the reduced form by the intervention of ascorbic acid, can be reduced in the liver to hydroquinone and subsequently conjugated to give glucuronate. The latter is excreted in the bile or can return to the systemic circulation to be further degraded in the kidney into α-tocopheronic acid and return to the systemic circulation, reaching the liver to be excreted in the bile (Drevon 1991).

Ascorbic acid, or Vitamin C, is a compound of carbohydrate origin frequently synthesized sufficiently to satisfy the basal need from the intestinal microbial flora of various animals, with the exception of mammals man and guinea pig (*Cavia porcellus*), which require to take it through the consumption of vitamin C rich foods (Sivaram *et al.* 2003). Ascorbic acid has a hydrophilic nature and is abundantly present both intracellular and extracellular; it is a reducing action compound capable of supplying electrons to oxidizing molecules, it intervenes on the free radical of Vit. E bringing it back to its reduced form (Bunker 1992). It can also act directly on H_2O_2 by reducing it to H_2O and O_2 (Padayatty *et al.* 2003).

Other compounds with antioxidant action of food origin are the molecules of the carotenoid family and polyphenols, even if their weight on the oxidative balance is marginal due to the low concentrations in the cellular components and particularly regarding the polyphenols as a result of the poor diffusibility between cellular and tissue compartments (Costantini *&* DellOmo 2006).

1.2.b - Enzyme complexes and endogenous origin antioxidant molecules.

An important scavenging action against free radicals depends on the presence of enzymatic complexes with antioxidant action, primarily superoxide dismutase (SOD) and glutathione peroxidase (GSH-Px). The O_2^- anion, of accidental and spontaneous formation following the transfer of electrons at the mitochondrial level in the process of formation of ATP, is converted into H_2O_2 by the intervention of oxidases (i.e. xanthine oxidase) and superoxide dismutase (SOD). H_2O_2 is less reactive than O_2^- and able to spontaneous diffusion through membranes, therefore more easily eliminable than O_2^-. The hydrogen peroxide is then in turn reduced in water and molecular O_2 by the intervention of GSH-Px and catalase (Birben *et al.* 2012); GSH-Px also intervenes in the reduction of fatty acid peroxides. The respiratory parenchyma is particularly rich in antioxidant enzymes, compensating for exposure to the highly oxidizing environment. The intestinal mucosa is rich in glutathione peroxidase, probably in contrast to peroxides of food origin (Birben *et al.* 2012).

A further and important antioxidant system is based on thioredoxin, a protein in whose active site are two cysteines with their respective thiol groups (-SH), with the ability to oxidize becoming two sulfide groups (-SO). This mechanism makes the molecule an efficient adapter; once oxidized, the active thioredoxin is regenerated by the action of thioredoxin reductase, using NADPH as electron donor (Mustacich & Powis 2000).

The liver parenchyma is also rich in the enzymatic systems in support of the detoxification processes to which it is responsible. All tissues and biological fluids contain enzymes with antioxidant action and at the intracellular level, the mitochondrial matrix of the cells is amply supplied with enzymes with an antioxidant action (Birben *et al.* 2012).

In addition to enzymes, the protection systems against radicals include numerous molecules with antioxidant activity, some of the endogenous production, others, as already seen, of food origin (Vertuani *et al.* 2004). Glutathione (GSH) is abundant in the cellular compartment and acts as an electron donor, by transforming into the oxidized form (GSSG), for degradation of H_2O_2 and lipid peroxides: this reaction can happen spontaneously or through the mediation of GSH-Px, for which GSH intervenes as a reduction cofactor (Curello *et al*. 1985). Consequently, GSSG is reduced in turn by glutathione reductase, with the intervention of NADPH as a source of e^-, and reported in the form GSH (Curello *et al*. 1985; Vertuani *et al.* 2004).

Another endogenous compound with important antioxidant activity is melanin, a lipophilic substance particularly present at the epithelial level, and able to cross the blood-brain barrier, Melanin is not a redox compound, and once oxidized it can no longer be reduced (terminal

antioxidant). Melanin plays an important protective role at the epithelial level against the ROs produced by electromagnetic radiation (Tan *et al.* 2000).

Ubiquinone or coenzyme Q10 is a fat-soluble benzoquinone involved in the transport of e^- in mitochondria and cellular membrane. It is particularly abundant in tissue with a high level of oxidative phosphorylation, as in the myocardial tissue. Ubiquinone intervenes as a free radical acceptor with reducing and membrane-stabilizing properties. It captures hydrogen ions (H^+) in the mitochondrial matrix and releases them into the intermembrane space. In addition, it intervenes in the function of enzymatic complex succinate dehydrogenase (SDH) allowing the oxidation of succinate and reoxidation of NADH, part of the Krebs cycle (Walker 1992; Birden *et al.* 2012).

Finally, uric acid (UA) appears to be an important reducing compound, especially in uricotelic species, acting as a terminal antioxidant. UA can directly react with O_2^-, splitting up in an irreversible reaction in 5-hydroxyurate, an unstable compound, with immediate subsequent conversion in allantoin, a soluble catabolite then excreted via the urine, interrupting the oxidative chain reaction. In mammals the oxidation of UA in allantoin also can be mediated from the enzyme Urate Oxidase, which involves UA, O_2, and H_2O determining the production of 5-hydroxyurate and H_2O_2,The latter immediately degraded by the catalase, but making the enzymatic oxidation reaction of the UA unfavorable from the oxidative balance point of view, as opposed to the spontaneous oxidation reaction (Simic *&* Jovanovic 1989).

1.3 - Oxidative stress and antioxidant systems in avian species

Birds appear to be particularly long-lived when compared to mammals, with an average life expectancy 3 times higher for the same body mass (Costantini 2008). The slow rate of aging contrasts with the high basal metabolism (twice higher than mammals, with a basal energy expenditure 15 times higher for the same mass), the high body temperature (on average 3°C higher than that of mammals with the same body mass) and the high glycaemic level (2-4 times higher), all characteristics probably justified by the high metabolic requirement associated with the high physical performance related to fly ability (Costantini 2008).

The marked longevity of birds could be explained by some biological peculiarities of the Aves class (Costantini *&* DellOmo 2006; Costantini 2008; Jones *et al.* 2008; Costantini 2011):

- Low ratio between ROS produced and oxygen use;
- High OS cellular resistance linked to ROS scavenging system particularly efficient;

- Mitochondrial and nuclear DNA low sensitivity to damage connected to the abundant presence of highly repeated non-coding sequences, with probable shielding action against ROS aggression;
- Cell membranes low unsaturation level, with less sensitivity to peroxidation events;
- high plasmatic levels of uric acid (UA) contributes to overall antioxidant capability. (Salmon *et al.* 2018).

However, the lack of positive association between antioxidant levels and longevity should be reported, an observation also supported by the failure to obtain an increase in longevity through the integration of antioxidants (Herbert 1994; Hulbert *et al.* 2007). It has been observed that in birds' antioxidant property and resistance to OS are highly variable between different species and this will likely affect their resilience (Cohen *et al.* 2007; Salmon *et al*. 2018). Rather, high levels of antioxidants in each species reflect an evolutionary and adaptive response to high levels of free radical exposure (Hulbert *et al.* 2007). Moreover, it was highlighted that within the same species there can be a great variation in terms of antioxidant capacity, mainly correlated with nutritional factors, and the antioxidants levels reduce the exposition to develop pathological situations (Costantini *et al.* 2008).

In the Aves class, among the most studied antioxidant factors, we find carotenoids, an alimentary xanthophyll being one of the fundamental pigments of the chromatic characterization of the livery of birds. From studies carried out the degree of pigmentation, proportional to the levels of carotenoids in the skin and appendages is positively associated with the choice of the reproductive partner by females in some avian species, probably associated with a good physical and sanitary performance of the male (McGraw 2005). Indeed carotenoids in birds contribute less than 0.002% of the total antioxidant potential (Costantini *&* Moller 2008), but it would appear to be directly correlated with the levels of other non-pigmenting food-borne antioxidant elements such as vitamins A, E and C. On the contrary, compounds are heavily involved in antioxidant processes in birds (Hartley *&* Kennedy 2004).

Ascorbic acid appears to be another important anti-redox compound taken with food and synthesized at the enteric level by the microbial flora (relevant in families with well-developed cecum intestines). In addition, it is synthesized at the renal level in numerous orders including *Galliformes*, *Pelecaniformes* (formerly *Herodioniformes*), *Anseriformes*, *Columbiformes*, *Falconiformes*, *Ralliformes*, *Cuckuliformes*, *Coraciformes*. In the *Psittaciformes*, *Stringiformes,* and *Piciformes* at the hepatic and renal level, as well as in the *Passeriformes,* with some exceptions for

the genera *Pycnonatus*, *Lanius*, *Aegithina*, *Pericrocotus*, *Acrocephalus*, *Rhipidura*, *Therpsiphone*, *Dicaeum*, *Aethopyga*, *Oriolus*, *Hirundo*, which do not synthesize ascorbic acid and depend entirely on that taken with the diet (Chaudhuri & Chatterjee 1969).

The Vitamin E complex determines an important protective action in cell membranes, but cellular presence and distribution in birds have never been investigated. It has been observed that administration within physiological ranges determines an improvement in the physical state and growth of the feathers if administered to growing chicks of *Hirundo rustica*, while higher quantities do not lead to improvements in the growth of the subjects, but at the same time any disturbance (Costantini 2008). It has also been experimentally observed that the supplementary administration of α-tocopherol in feed ration in chickens exposed to thermal stress improves their immune response (IgG and IgM increase) following an antigenic stimulus, compared to the control group (Sandhu *et al.* 2012).

Glutathione is an important antioxidant factor in birds, particularly abundant in all organic fluids of the extracellular compartment, including plasma, intercellular fluids, and seminal fluid (Isaksson *et al.* 2005).

Uric acid, the final result of the catabolism of purines in the *Aves* class, is considered another potential important antioxidant (Tsahar *et al.* 2006; Cohen *et al.* 2007; Horak *et al*. 2007; Salmon *et al*. 2018). In uricotelic animals such as birds, nitrogen excretion occurs in the form of uric acid, which is circulating at concentrations four times higher than those found in mammals.

Finally, also in birds, the enzymatic complexes with antioxidant action play a decisive role in oxidative scavenging phenomena, in particular, the SOD and the GSH-Px complex have been studied and highlighted in numerous works concerning avian species (Wiersma *et al*. 2004; Min *et al*. 2018, Surai *et al.* 2018).

1.4 - Consequences of oxidative stress on organisms

The state of OS occurs when the production or introduction of free radicals exceeds the capabilities of the organic antioxidant systems or if the latter undergo a reduction below sufficient minimum level (Birden *et al*. 2012). More specifically, it can derive from increased production of pro-oxidants, a decrease in antioxidants, a depletion of the metals cofactor for enzymes with antioxidant activity (i.e. Se, Mg, Zn), or as a result of deficits in repair or reconstitution processes of antioxidant systems (Costantini 2008).

The increase in oxidative levels involves a denaturing action on cellular chemical constituents, compromising the functionality of biological compounds (Gershman *et al.* 1954) and accelerating the cellular aging process (Harman 1956), inducing cellular type responses defensive including modulation of genetic expression, leading to the activation of metabolic processes and the production of enzymes aimed at increasing the antioxidant potential (Dalton *et al.* 1999).

Nucleic acid, especially DNA, can be attacked and denatured in all its fractions with a consequent predisposition in the evolution of neoplastic forms, in cellular aging, in nervous and cardiac degeneration, and the evolution of autoimmune diseases (Ghosh *&* Mitchell 1999). The attack does not only involve DNA damage but also the lighting of transcription factors activating metabolic pathways aimed at reducing oxidants (i.e. antioxidant enzymes). For example, some of the aforementioned sequences are rich in GC fractions particularly susceptible to oxidation: in the event of aggression they determine the exposure of 8-OH-G with a consequent increase in affinity for transcription factors and therefore an increase in relative gene expression (Ghosh *&* Mitchell 1999).

Another way of activation of the "Redox sensitive" transcription factors is associated with the GSH/GSSG ratio, one of the main indicators of OS levels; in physiological conditions, it has a clear advantage due to the non-oxidized form of glutathione, in the event of an increase in the oxidized form GSSG at the intracellular level, it involves the activation of transcription factors and the consequent metabolic chain (Poli *et al*. 2004).

Highly attackable compounds are lipids, involving the peroxidation with consequent loss of integrity of the phospholipid membranes and inactivation of membrane proteins until it gets to the complete suppression of cellular functions (Girotti 1998).

ROS action on the peptides is of the denaturing type, with possible disruption of all structural levels of proteins, with enhanced sensitivity of the sequences rich in sulfured amino acids (Met, Cis), due to the presence of thiol groups; the consequence is the loss of biological functionality of the peptides involved (Kelly *&* Mudway 2003).

All these events, overall, involve a loss of functionality and degeneration of the interested cells until it gets to induce, based on the molecular structures involved, mutagenic action, induction of apoptosis, or cell necrosis. The damage is reflected on tissue and therefore the whole organism, with alteration entity directly proportional to the level of exposure of the organism to pro-oxidant factors, where generally the organs most subject to OS damage are -emunctories, such as liver and kidneys, involved in the ROS elimination first front line, again tissues with high metabolic

consumption (such as the myocardium) and those with high levels of lipids, such as the brain (Dalle-Donne *et al.* 2006).

1.5 - Oxidative stress assessment degree.

The degree of OS for an organism depends on the balance between the action of innumerable antioxidant systems (molecules and enzymes) and the oxidative aggression potentially supported by numerous oxidizing factors capable of attacking practically all organic compounds and determining the formation of many possible types of free radicals, in variable proportions, both intra and extracellular (Costantini 2008). It follows that for the assessment of the degree of OS it isn't sufficient to measure a single biomarker, but it is essential to consider a pool of indicators, including free radicals and compounds produced by the oxidation of biological molecules, evaluating them in correlation with enzymatic and non-enzymatic antioxidant factors (Finkel *&* Holbrook 2000; Costantini *et al.* 2006; Costantini 2008).

Although it has been observed that the biomarker levels of OS in different tissues are generally positively correlated, it should be reported that the correlation levels may vary from tissue to tissue and from species to species, also in a relevant way (Surai 2002). Based on this, it can be said that plasmatic levels can be broadly indicative of the general OS state, but it would be too useful to evaluate the level of correlation between plasmatic and tissue oxidative state (Surai 2002; Costantini 2008). Said this, a clinically useful biomarker should be able to meet the following criteria: show specificity for a certain disease (diagnostic) and have prognostic value (Frijhoff *et al*. 2015). Again, to be clinically useful, a biomarker must also be reasonably stable, present in an easily accessible tissue minimally invasive to take, cost-effective to measure and so reproducibly on a large scale (Frijhoff *et al.* 2015).

Levels of tissue or, more often, plasma free radicals are among the most commonly evaluated biomarkers, providing a direct indication of the degree of exposure to oxidative action by tissues, among these the most frequently measured are the level of determinable reactive oxygen metabolites (d-ROMs), which are considered a reliable measure of the concentration of plasmatic global reactive species related to oxygen (Trotti*, et al.* 2002), or the plasmatic level of superoxide anion (O_2^-), of hydroxide peroxide level (H_2O_2), of hydroxyl radical (-$OH^\bullet$), or of nitric oxide (NO) (Christou *et al*. 2003).

In addition to measuring free radicals, the levels of the compound released as reaction products following the organic molecules oxidative attack can be investigated: these have the advantage of

indicating the biological molecules damage degree, consequently they provide information on the cellular and tissue damage extent. Among these compounds is malondialdehyde (MDA), a reactive aldehyde that forms after polyunsaturated fats peroxidation, indicative of oxidative damage to cell membranes (Del Rio *et al.* 2005).

Uricotelic species, as birds and reptiles, are devoid of urate oxidase, therefore enzymatic degradation of UA into allantoin does not occur, nevertheless allantoin can form only following direct oxidation of uric acid by free radicals, making allantoin in these species a potential good biomarker of OS (Tsahar *et al.* 2006).

Fundamental biomarkers of OS are compounds with antioxidant action, both of food origin, such as carotenoids, vitamin E, and ascorbic acid, and of endogenous origins, such as glutathione (GSH). These can be investigated directly, by measuring their plasma or tissue concentration, or indirectly, by evaluating the antioxidant potential that these confer on plasma or tissues. This is the case of ferric reducing antioxidant power (FRAP) or even the plasmatic antioxidant test (PAT), methods that allow quantifying the plasma antioxidant potential associated with non-enzymatic compounds (Huang *et al.* 2005). In addition to compounds with antioxidant action, the study of the activity of enzymes or enzymatic complexes with antioxidant action is often examined concerning other biomarkers, among the most studied are superoxide dismutase (SOD), glutathione peroxidase (GPx), and glutathione disulfide reductase (GSR) (Christou *et al.* 2003).

Cap. 2 – Immune response and oxidative stress in birds: possible diagnostic applications

In the last years, a growing interest has risen in the study of OS in the clinical field (both in human and veterinary medicine); OS is considered a reliable index of direct biomolecular damage to nucleic acids, proteins, and lipids, as well as a marker of inflammation. (Dalle-Donne *et al*. 2006; Birden *et al.* 2012). Moreover, OS plays an important role in the evolution of acute diseases (such as in poisonings and irradiation syndromes), as well as in the development of chronic pathological conditions (such as chronic respiratory pathologies, cardio-circulatory, and neoplastic diseases) (Dalle-Donne *et al.* 2006).

Based on these assumptions in the clinical setting, various research initiatives have investigated the correlation between OS, estimated through the evaluation of various OS biomarkers, and stated the predisposition to given pathologies, pathology frameworks, and in correlation to certain immune states. Most of the research concerned human medicine (Dalle Donne *et al.* 2006; Frijhoff *et al.* 2015), more limited are the information about the veterinary field, even less when it comes to avian

physiology and pathology (Colitti *et al.* 2019). Speaking of clinical purpose, the application of the study of OS concerning the immune response turns out to be particularly interesting, both from the perspective of indirectly investigating the immune status at the time of sampling and for prognostic purposes, to predict the trend of the immune response, in a given subject, following a given immune stimulus (Colitti *et al.* 2019; Iemmi *et al.* 2021).

The immune system of birds presents several similarities to that of mammals from a cellular and functional point of view. Even in birds, antigen-presenting cells -among which are tissue macrophages, histocytes, and thrombocyte play a primary role- engulf and process the antigen, exposing it to lymphocyte cells (Maouia *et al*. 2020). Following the immune cascade activation, the immune cells (macrophages, eosinophils, heterophils, B and T lymphocytes) carry out their pathogen-killing activity, in large part by producing and releasing pro-oxidant compounds as O_2^-, H_2O_2, hypochlorous acid (HOCl), peroxynitrite ($ONOO^-$), hydroxyl ($-OH^\bullet$) and ozone (O_3). This, however, progressively affect the organism's redox balance accordingly (Hampton *et al.* 1998).

Over the aggression action on the antigen, the redox-active compounds also intervene on host cell through a biphasic action based on concentration. At a low concentration, well below levels manageable by the anti-ROS systems, they exert a modulatory cellular action, working in the intercellular communication (as macrophagic NO in the initial phase of leukocyte recruitment). Contrarywise, the increase to high concentration, higher than the levels manageable by the anti-ROS mechanisms, cause host cellular and tissue damage, including a harmful action to immune system cells themselves, with a potential negative impact on immunity response progression and efficacy (Costantini *&* Møller 2009). This way, redox environment, determined by the interaction between pro-oxidant and antioxidant components, affects the evolution of the immune response. (Sorci & Faivre 2009).

2.1 – Immune activation and oxidative stress in birds; exposure to *Mycoplasma gallisepticum* antigens as a possible study model.

It is known that the immune response both influences and is influenced by the oxidative state of the subject, but very little is known about the interdependence dynamics of these two aspects, particularly in the field of avian physiology (Costantini & Møller 2008; Colitti *et al*. 2019).

To investigate the interrelation between immune response (IR) and OS, it would be necessary to monitor in parallel the trend of IR and OS indicators following exposure to an immune stimulus, such as an antigenic stimulation. In the avian field, various tests of administration of lipopolysaccharides (LPS) were conducted on *G. gallus*, in which a marked positive correlation was observed between levels of exposure to LPS and indicators of oxidation and vice versa negative with the levels of antioxidants, but not special insights were made on immune response indicators (Zheng *et al.* 2016) Numerous observations have also been made regarding the exposure of specimens of avian species to pathogens including *Salmonella spp.*, *Staphylococcus aureus, Serratia marcescens*, *Aspergillus spp*. (Winkelstein *et al*. 2000; Dinauer 2005), Newcastle Disease Virus, Avian Influenza Virus, Avian Reovirus, Duck Hepatitis Virus, Infectious Bronchitis Virus, Marek's Disease Virus, Avian Leukosis Virus (Rehman *et al.* 2018), highlighting in all cases a pro-oxidant action, both direct, caused by the metabolism of pathogens, and indirect, resulting from the alteration induced by the infection in the cellular metabolism of the host, also affecting the immune cell lines. Also in these cases, the study of the trend of the immune response in parallel to the oxidative one has not been further discovered.

Wanting to study the effect of exposure to an antigen on the immune response concerning the oxidative response in avian species, an interesting candidate could be *Mycoplasma gallisepticum* (MG), a bacterium without a cell wall and falling within the *Mollicutes* class, one of the main agents of avian mycoplasmosis, an extremely frequent disease in *Gallus gallus* found in both intensive and rural/amateur farming. The infection is characterized by high morbidity associated with variable mortality, depending on health conditions of the subjects and possible concomitance of other diseases and/or health deficits. The pathology manifests itself in its full-blown form with respiratory and urogenital symptoms, reduction and alteration of laid eggs, and embryonic death associated with the hatching of a high percentage of sick chicks. In many cases, however, the infection is characterized by a long incubation period. Symptoms manifest following immune deficiency. Likewise, it is not uncommon for subjects to survive the clinical evolution to subclinical or chronic forms of this pathology. After symptoms resolution birds may remain excretors for very long periods (Nascimento *et al*. 2005; Umar *et al.* 2017).

Pathogens belonging to *Mycoplasma* order are particularly insidious due to their ability to interfere with the host's immune system: this phenomenon is associated with the presence of surface antigens with mimetic action and the property of producing factors capable of altering the leukocyte

response. They can stimulate immune response from macrophages, monocytes, T-helper cells, and NK cells, inducing the synthesis of cytokines such as tumour necrosis factor (TNF-α), interleukin (IL-1, 2, 6), and interferon (IFN-α, β, γ) going to increase the inflammatory response and leukocyte function, promoting excessive recruitment and apoptosis (Razin *et al*. 1998). They are also able to produce ROS such as hydrogen peroxide (H_2O_2) and superoxide anion (O_2^-), with a consequent local increase in oxidative activity and subsequent cell and tissue damage (Nascimento *et al*. 2005).

Usually, the clinical management strategies of MG outbreaks tend to minimize the use of antibiotics, since these are food-producing livestock species and in consideration of pathogen's high tendency to show drug resistance. Therefore, enforcement actions are normally based on biosecurity, health and hygiene interventions, good management practices, continuous monitoring and removal of infected flocks, farmer awareness, and tight vaccinal protocol (Shoaib 2021). Vaccination employing deactivated or attenuated vaccines and the consequent immune response that follows against MG is particularly effective in reducing the pathological effects and reducing the risk of reinfection of immunized subjects (Nascimento *et al.* 2005). This has been suggested even if the establishment of protective vaccination coverage is not a certain consequence of the treatment, but it is strictly conditioned by an efficient immune response (Zimmermann *&* Curtis 2019).

Cap. 3 – Heavy metals and oxidative stress

3.1 - Heavy metals, considering HM and THM

For heavy metals (HMs) we mean those with an atomic weight higher than the molecular weight of water (Fergusson 1990), all of which are part of the transition metals. We are specifically speaking of: aluminium (Al), iron (Fe), silver (Ag), barium (Ba), beryllium (Be), cadmium (Cd), cobalt (Co), chromium (Cr), manganese (Mn), mercury (Hg), molybdenum (Mo), nickel (Ni), lead (Pb), copper (Cu), tin (Sn), titanium (Ti), thallium (Tl), vanadium (V), zinc (Zn), and some metalloids with properties similar to those of heavy metals, such as arsenic (As), bismuth (Bi) and selenium (Se). These are elements that in aqueous solution have a tendency to hydrolyse forming cations, some of these intervene in metabolic reactions as coenzymes or components of functional macromolecules, including Fe, Zn, Cu, Se, Co, Mb, Mg. Based on their biological role they are counted among the microelements, which are indispensable to living organisms at micro-concentrations in the order of µg or mg day based on the element and the organism. Also, they carry out toxic activity in the event of exposure to high quantities (Fergusson 1990).

In addition to the aforementioned HMs, some elements are defined as toxic heavy metals (THMs) including Cd, Ni, Pb, As and Hg. For these elements a biological function has never been demonstrated, but they are capable of interfering on metabolic processes, both following exposure to high concentrations causing acute toxicity (in the order of mg for kg body weight) and as a consequence of prolonged exposure to micro concentrations, resulting in subacute or chronic toxicity pictures (Singh & Kalamdhad 2011; Duffus 2002). HMs are naturally present in the environment at variable concentrations primarily depending on the composition of the mineral composition of the habitat and the degree of its deterioration. The increase in environmental contamination levels depends in most cases on the anthropogenic release of polluting compounds (Singh & Kalamdhad 2011). Among the anthropogenic sources most involved are industrial activities (fumes and waste), agricultural activities (pesticides, herbicides, and synthetic fertilizers), wood processing and treatment, industry of electronic components, and the degradation of paints and plastic compounds (Kaur *et al.* 2012).

The toxic action of THMs occurs at all levels of the food chain: starting from the soil microbiota, to plants, to the most complex organisms up to the apex of the food chain (Singh & Kalamdhad 2011). In general, they appear to be easily assimilated even at low concentrations, primarily through ingestion, but also by inhalation (metal micro powders) or transcutaneously, as demonstrated for Pb (Engwa *et al*. 2020). After absorption, the HMs are distributed by the circulatory blood stream (usually binded to plasma proteins) in the various parts of the body, concentrating in target organs based on the metal, causing in some cases the phenomenon of biomagnification along the food chain, as for Hg and Cd mainly in aquatic ecosystems (Diez *et. al* 2000). The excretion of these elements generally occurs via the hepato-biliary or renal route, concentrating and often causing damage to the excretory organs themselves (Engwa *et al.* 2020).

3.2 – Mechanisms of HM toxicity

The exposure of an organism to high concentrations of HM in short periods determines phenomena of acute toxicity, firstly due to the high capacity of these elements to induce the formation of ROS following the iteration with biological compounds. Consequently, cell and tissue damage mainly localized to the excretory organs, liver, and kidneys, in which these substances tend to concentrate (Ibrahim *et al.* 2006). Neurological symptoms are often associated with acute Pb intoxication, because of the competitive action against Ca^{2+}, with consequent interference in the release of neurotransmitters (Konur & Ghosh 2005). Essentially situations of acute THM toxicity are limited to

episodes, while contrarywise the phenomena of chronic toxicity, associated with prolonged exposure to reduced quantities of THM, are extremely widespread and often underestimated. The consequences on the organism derive from the ability to induce oxidative aggression of cellular structural components, interference with enzymatic and receptor complexes and interference with nucleic acids (Valko *et al.* 2005; Gall *et al.* 2015).

HMs interference action on enzymatic and receptor complexes can occur consequently by direct competitive action against compounds or ions with regulatory activity, by inducing the production of ROS and consequently denaturing action on functional protein. The neurotoxic action of Pb can impair learning and memory in the brain by inhibiting the N-methyl-d-aspartate receptor (NMDAR) in the hippocampus (complex involved in the molecular learning process) and can block neurotransmission by inhibiting the release of the neurotransmitter, blocking the calcium channels (Ca^{2+}) responsible for the release of GABA and glutamate at the hippocampus level (VGCC) and the reduction of neuronal expression of the brain-derived neurotrophic factor (BDNF) (Engwa *et al.* 2019). Lead occupies the sites of the Ca^{2+} channels, preventing the entry of Ca^{2+} into the cells and therefore the consequent release of the neurotransmitter (Konur & Ghosh 2005).

The interaction of HM with organic compounds can determine the formation of ROS through various processes, typical for each HM. ROS, following their formation, determine a direct molecular aggression with consequent loss of functionality and molecular integrity (Valko *et al.* 2005). At a biological level, Fe, Cu, Cr, V, and Co ions, undergo oxidation by O_2^-, and following the interaction with H_2O_2 due to the Fentom reaction, determine the production of hydroxyl radical ($R\text{-}OH^-$). The Hg, Cd, and Ni intervene by depleting the glutathione available in the cells and binding to the sulfhydryl groups present in the proteins, denaturing them and inducing production of ROS . The protein compounds most subject to denaturing are generally those with more complex tertiary and quaternary structures, rich in thiol groups, including most of the enzymatic complexes. These are attacked and inactivated, seriously damaging cellular function (Ibrahim *et al*. 2006).

Highly susceptible to HM effect are membrane phospholipids, with consequent formation of lipid radicals, and subsequent chain reaction and further formation of lipid peroxide radicals. The main aldehyde product of lipid peroxidation induced by the initial intervention of heavy metals is malondialdehyde (MDA), particularly useful, from the analytical point of view, as a biomarker of lipid peroxidation consequent to HM exposition (Valko *et al* 2005).

Some heavy metals are known to have a carcinogenic effect by interfering with the activity of proteins involved, in apoptotic activation, DNA repair, DNA methylation, cell cycle regulation, cell growth, and differentiation (Kim & Koo 2007). Furthermore, the carcinogenic effects of some heavy metals have been related to the activation of redox-sensitive transcription factors such as AP-1, NF-κB, and p53, through electron recycling via an antioxidant network. These transcription factors control the expression of protective genes that induce apoptosis, stop the proliferation of damaged cells, repair damaged DNA, and regulate the activity of immune system cells (Valko *et al.* 2005). Interference of HMs with transcription factors AP-1 and NF-kB was observed in mitogen-activated protein kinase (MAP) pathways, in which nuclear transcription factor NF-kB is involved in controlling inflammatory responses, while l 'AP-1 is involved in cell growth and differentiation (Valko *et al*. 2005). The p53 protein is important in cell division as it protects a cell cycle checkpoint and controls cell division (Chen 2016). Inactivation of p53 allows for uncontrolled cell division and thus destruction of the p53 gene has been associated with most human cancers. Furthermore, the AP-1 and NF-kB family of transcription factors are involved in both cell proliferation and apoptosis, and regulate p53. ROS, generated by HMs within the cell, selectively activate these transcription factors, thus may suggest that cell proliferation or cell death may be influenced by exposure to carcinogenic metals (Valko *et al*. 2005; Chen 2016).

Among the HMs with mutagenic carcinogenic action, there are also Pb, Hg, Ni, and Cd. It has been suggested that the carcinogenic activity of Pb can be carried out through indirect damage, through the formation of ROS with consequent oxidative aggression to DNA and the repair systems of nucleic acids. In addition, Pb can interrupt transcription processes by replacing zinc as a coenzyme in some regulatory proteins (Silbergeld *et al.* 2000).

The carcinogenic action of mercury seems to occur through the generation of free radicals and consequent OS increment, thus damaging the biomolecules, with consequent oxidative action on DNA and repairing complexes (Crespo-Lopez *et al.* 2009).

Nickel can induce carcinogenesis through the formation of free radicals and consequently damaging nucleic acids and repair systems (Zambelli *et al.* 2017), or through direct interference with transcription factors and with the controlled expression of some genes. Nickel can promote methylation and induce down-regulation of maternally expressed gene 3 (MEG3), thereby increasing hypoxia-inducible factor 1α, two proteins that are known to be implicated in carcinogenesis (Zhou *et al*. 2017).

Cadmium interferes with the modulating processes of apoptosis, by the production of free radicals, by intervention in DNA methylation, and by direct damage to DNA (Engwa *et al.* 2019).

3.3 – HMs and ecotoxicity: eco-monitoring strategy

Even though THMs are naturally present in the environment at variable concentrations, the increase in the levels of environmental contamination by THMs is mostly due to the anthropogenic release of polluting compounds (Gall *et al.* 2015). These sources of THMs mostly include fumes and wastewater produced by industrial activities, pesticides, herbicides, and synthetic fertilizers employed in agriculture, timber processing and treatment, degradation of paints and plastic compounds, and consequently processing of electronic disposal components (Duffus 2002).

Moreover, THMs are persistent pollutants and are subjected to biomagnification, which means that they tend to accumulate progressively in the body of animals and along the food chains (Singh *&* Kalamdhad 2011). The toxic action of THMs occurs at all levels of the trophic chain, starting from the microbiota of the soil, through plants, up to the most complex organisms at the top of the food chain (Gall *et al*. 2015), also reaching the trophic resources exploited by human society and consequently reaching an impact on human health (Liang *et al*. 2019). In consideration of the potential danger for the ecosystem and public health represented by the exposure to THMs, the development of strategies aimed at monitoring THM levels in the environment is of the utmost importance (Martin 2012). The measurement of the THM levels directly from the environmental abiotic matrix (water, soil, air) is not considered an accurate or suitable method, because environmental levels can vary in time (especially in the water or in the air), and thus a sample showing low levels cannot exclude the risk of dangerous exposure to THMs; by contrast, THMs in the mineral matrix may not be necessarily transferred to the trophic chain. Indeed it is common for the mineral matrix to include THMs among its constituents, but often these are firmly incorporated in the mineral molecular structure, minimizing their accessibility to biological activity (Valko *et al*. 2005; Singh *&* Kalamdhad 2011). This has led to the development of indirect monitoring systems, such as measuring the levels of THMs in bio-indicator species, to evaluate the amount of these elements in the ecosystem, with the ultimate goal of protecting public health, the integrity of the ecosystem, and can plan and put in place effective countermeasures (Singh *&* Kalamdhad 2011). An ideal bioindicator species should be widespread, easily sampled, and not excessively sensitive to the compound under study, since in that case, even low-level exposure could lower the local population, thus hindering monitoring activity (Valko *et al.* 2005).

Carnivore and scavenger birds are at the top of the food chain and are therefore particularly exposed to bioaccumulation of toxic compounds present in the ecosystem (Bond & Lavers, 2011). For this reason, these animals are considered as good environmental pollutant bioindicator (Nardiello *et al.* 2019), allowing to investigation the presence of contaminants along the food chain, environmental contamination levels, and the effects on animal and human health (Espin *et al.* 2014).

Furthermore, a suitable substrate to be taken from bioindicator species should be easily collected in the field, with minimal damage to animals; birds are often studied as bio-indicators, and the covering feathers can be collected by operators lacking the skill needed to perform blood sampling, with no or very little discomfort for the animals (Martínez et al. 2012).

THM levels detected in feathers may reflect the levels in the blood at the time of feather formation (Holt & Miller 2010; Bond & Lavers 2011) indicative of exposure to THMs in a relatively short period. In addition, THMs present in the environment deposit on the surface of feathers during the period between feather formation and sampling (Nardiello *et al.* 2019), or due to preening activity (Markowski *et al*. 2013). Indeed, the measurement of Pb in the feathers of young herons was found to be a reliable indicator of the level of exposure to metal in the time frame relative to the eruption of the feather and was found to be correlated with the metal concentrations in kidneys and bones (Goede & De Bruin 1984)

Several previous works have taken into consideration the measurement of THMs in feathers of wild birds to evaluate environmental pollution levels. THM levels have been assessed in feathers of songbirds as *Cyanistes caeruleus* and *Parus major* (Dauwe *et al.* 2000; Veerle *et al*. 2004), in birds of prey as *Accipiter nisus*, *Aquila heliaca*, *Buteo buteo*, *Athene noctua*, *Tyto alba* and *Stryx aluco*, (Denneman & Douben 1993; Dauwe *et al.* 2003; Pain *et al.* 2005; Naccari *et al.* 2009), in herons as *Nycticorax nycticorax* and *Ardea cinerea* (Golden *et al*. 2003; Kim & Koo 2007), in corvids as *Pica pica*, *Corvus splendens* and *Corvus corax* (Dmowski & Golimowski 1993; Giammarino *et al.* 2014; Janaydeh *et al.* 2016; Janaydeh *et al*. 2017) and in the end in pigeons (*Columba livia*) (Frantz *et al*. 2012).

Cap. 4 - Objectives of PhD project

The THMs level assessment in sentinel species tissues is an already widely consolidated method for the indirect monitoring of the environmental contamination (Golden *et al*. 2003). Unfortunately, however, the high complexity and cost of the analysis (analysis method in ICP-MS) limits it

applicability in extended and prolonged monitoring plans. For this reason, in our project we investigated the use of OS biomarkers as indirect indicators of exposure to THMs, in hope of highlighting a method economic, rapid, minimally invasive, and sensitive to early highlight phenomena of exposure to THMs.

Specifically, in the present work, we assessed levels of Cd, Ni, Pb, and Hg in the feathers of a sedentary bird species, the Eurasian magpie (*Pica pica*), which is a widespread omnivore corvid, with opportunistic predatory and scavenger habits. Magpies are synanthropic sedentary birds, characterized by a reduced home range, (between 10 and 50 km), living in urban and rural areas, both on the plains and the hills (Madge *&* Burn 1994). For these reasons and accordingly to its top food chain niche (Burger 199). It is a local species considered an ideal subject for the biomonitoring of heavy metal pollution (Dmowski *&* Golimowski 1993). Parallel to the measurement of THMs levels we measured the biomarkers of OS at the plasma level, specifically by assessing the levels of MDA, NO, SOD, d- ROMs, FRAP, and O_2^-. Finally, we investigated the interaction between levels of THMs and OS biomarkers highlighted.

The PhD work was extended also by examining the interrelation between OS and immune response in two target avian species, the domestic chicken (*Gallus gallus domesticus*), and a wild species, the Eurasian magpie (*Pica pica*). More specifically, two studies were conducted.

In the first study, OS was evaluated by assessing biomarkers in birds' blood, considering d-ROMs and the circulating non-enzymatic antioxidant compounds, using the PAT. In addition, lipid peroxidation levels were assessed considering MDA levels. OS biomarker levels were then compared in parallel with immune response indicators, by studying white blood cell count (WBC) and differential leukocyte examination considering the populations of lymphocytes (Lym), monocytes (Mon), heterophiles (Het), eosinophils (Eos), basophils (Bas) and thrombocyte (Thro).

In the second study, a group of Australorp bred hens were examined, where immunity and OS state were monitored following the intraocular administration of vital *Mycoplasma gallisepticum* vaccine strain, considering for the monitoring of OS state the trend MDA, NO, SOD, d-ROMs, FRAP, and O_2^- levels. The immune response was monitored by studying WBC and differential leukocyte evaluation examined in correlation with the trend of plasmatic anti-MG antibody titre.

In the last phase of these two studies, the correlation between OS levels and immunity index was investigated, to evaluate a possible clinical utility of OS assessment in avian species, as a tool for early diagnosis of subclinical pathological conditions and animal welfare monitoring.

MATERIALS AND METHODS

Cap. 5 - Materials and methods concerning the study of Feathers Heavy Metal contamination in relation with OS state in Eurasian Magpies (*Pica pica*)

5.1 – Animals and sample collection

Magpies (n = 64) were captured from January to November 2019, in 8 capture sites distributed in the province of Parma (Italy). The capture sites were selected in consideration of the level of urbanization, considering the distance from urban/industrial areas as an indicative factor of the presumed level of environmental pollution. Magpies captured in the 4 sites located within 1 Km from urban areas and manufacturing or industrial activities, were identified as urban zone (UZ) groups, while those captured in the 4 sites farther than 5 Km from urban activities were identified as rural zone (RZ) group.

The study was approved by the Emilia Romagna Regional Ethical Committee (ISPRA Prot. 8093/T-A31 of February 21st, 2019 and Region ER Det. 3751 of January 3rd, 2019). The authorization included trapping of target species, capture modality (with lure birds using 10 no-kill Larsen traps), and all subsequent measurements, marking, and sampling procedures. All captured magpies were considered clinically healthy evaluated for biometrical measurements and marked with numbered tarsal open rings. During the capture activity, from each subject was taken a 1,5ml blood sample from the right jugular vein. The blood sample was stored in 1ml heparinized tubes and refrigerated at 8° C until delivery to the laboratory, where 0.7ml was used for blood Hg research. The remaining blood was employed for plasma separation and the further research of OS biomarkers.

In addition to blood, a sample of feathers from each subject (2 external helmsman and about 10 covering feathers) was collected for the analysis of HM levels. All birds were released after sampling and marking operations.

5.2 – HM levels assessment

The evaluation of Cd, Ni, Pb, Hg levels was performed through the inductively coupled plasma mass spectrometry technique (ICP-MS). To remove the external contamination from the surface of the feathers, a sequential washing process was performed before analytical determination. In the first step tap water was used, followed by distilled water, Milli-Q water and the last step was acetone acetone (Dauwe *et al*. 2003; Veerle *et al.* 2004; Pain *et al.* 2005). A microwave-assisted acid

digestion procedure, adapted from previously published techniques (Fromant *et al.* 2016) was carried out to obtain metal contents. 0.5 g aliquots of each sample were weighed into Teflon PTFE flasks and 1.5 ml of a freshly prepared mixture of concentrated HNO_3 (69%) and H_2O_2 (30%) in a 3:1 v/v proportion were added. The flasks were closed and left to predigest for 12 h at room temperature. The flasks were then sealed, and microwave digested (15 min with constantly increasing temperature up to 180 °C, and finally 5 min at this maximal temperature). Once the digestion was completed, the flasks were allowed to cool down to room temperature, and the mixture was diluted to 10 ml with deionized water. A blank digest was carried out in the same way to provide a negative control. All sample solutions were clear. To avoid losses of volatile elements, the second set of identical samples from the same individuals was dried at 80°C in an oven until they would reach constant weight to calculate the percentage of humidity in each sample (average humidity of 20.85% in feathers). The accuracy of the microwave digestion method was checked by standard reference material (BCR® certified reference materials - ref. 185R, Community Bureau of Reference, EU). Four replicates were done on NIST SRM 1577b Bovine liver to check the accuracy, and the results were in good agreement with the certified material, with a mean recovery rate of 85-102%.

A platform collision ICP-MS 7900 equipped with an integrated autosampler (Agilent Tech) was used for element detection. For an optimal nebulization of the sample, a Peltier-cooled (2°C) cyclonic chamber (Elemental Scientific, Omaha, NE, USA) and a low-flow (0.25 mL/min). Mein hard concentric nebulizer (LGC, London, UK) was employed. Both the collision gas and the argon for the plasma have a purity of 99.999% and have been supplied by Praxair (Madrid, Spain).

Each day, the ICP-MS was calibrated to obtain the highest values of intensity indicated by the ratios CeO/Ce < 2.5 %, Ce^{++}/Ce < 3 % and background (220) < 1 cps. The instrumental detection limits were 0.005 mg/kg for all the elements. Calibrating solutions were prepared daily from a 10 mg/L Multi-Element Calibration Standard 3 solution (PerkinElmer, Inc., Shelton, CT). The same certified sample of lyophilized bovine liver previously indicated was used for quality control of the analytical procedure. The limit of detection (LOD) and limit of quantification (LOQ) were determined according to the ICH-Q2 guideline on method validation (ICH 2005), after analysing repeated blanks with the same procedure used for the samples and determining the standard deviation. The final values of both parameters were calculated considering the samples' dilution factor and the weight and were in all cases lower than 0.003 and 0.009 mg/kg for LOD and LOQ, respectively. The coefficients of

variation for replicate samples (n = 5) were determined to be lower than 5.3%. All samples were run in batches that included analytical blanks.

For the research of blood Hg, an aliquot of 0.7 g of whole blood was transferred to a 50 ml Digi-Tubes for subsequent digestion with Digi-Prep (SCP-Science) mode. 10 mL of concentrated nitric acid was slowly added to the sample tubes, allowed to stand for 15 minutes at room temperature, and then subjected to mineralization at 75° C ± 10 ° C overnight. The samples were cooled down and 20 mL of demineralized water (HPLC grade) was added to each tube: the solution was sealed and vigorously shaken for 5 minutes. Then 1 mL of solution was added to 10 mL of 2% aqueous nitric acid solution with 0.5% hydrochloric acid. The solution thus obtained was used to measure ICP-MS, following the procedure already described for the analysis from feathers.

Metal concentrations were expressed as mg/kg dry weight (d.w.) since dry values are more reliable and consistent compared to wet weight values (Adrian *&* Stevens 1979).

5.3 – OS biomarkers determination

Plasmatic biomarkers of OS were evaluated in all the subjects, to establish a possible correlation between S/P ratio, leukocytes levels, and OS biomarker. Specifically, were assessed the levels of MDA, d-ROMs, O_2^-, NO as an indicator of oxidative events. Also, enzymatic antioxidant capacity was evaluated by SOD analysis, while scavenging non-enzymatic activity was determined by means of FRAP assay. These biomarkers were chosen because they are commonly employed to evaluate OS in most species, including birds (Christou *et al*. 2003).

Lipid peroxidation was assessed by measuring thiobarbituric acid-reactive substances according to a previously published method (Zeb *&* Ullah 2016). It is expressed as nmol/ml of MDA. Briefly, 100 µl of plasma were added to 200 µl of a solution composed of 25 ml of HCl 1N, 75 ml of distilled water (HPLC grade), 380 mg of thiobarbituric acid, and 15 g of 15% trichloroacetic acid; then, 5 µl of a 0.2% 2,6-ditert-butyl-4-methyl-phenol solution in 95% ethanol were added. The mixture was then vortexed and heated at 80° C for 15 min. The solution was then centrifuged for 10 min at 6000 rpm, and the supernatant was collected and examined with a spectrophotometer at a wavelength of 532 nm (Varian Cary UV-Vis 50, Varian Technologies Italy, Milan, Italy). The calibration curve was constructed employing increasing MDA concentrations (7.8, 15.6, 31.3, 62.5, 125, 250 nmol/ml).

d-ROMs were assessed through a commercial test, following the manufacturer's instructions (d-ROMs Assay, Diacron s.r.l. Grosseto, Italy) (Trotti *et al*. 2002).

Anion O_2^- levels were measured by the WST-1 test (WST-1 Assay Reagent, Sigma Chemical Co Lt, St. Louis, MO, USA) according to the guidelines of the manufacturer. The assay is based on the reducing capacity of superoxide for highly water-soluble tetrazolium salt, giving rise to a soluble formazan that can be quantitatively evaluated by a colorimetric assay (Xu *et al.* 2016).

NO was assessed by a colorimetric technique measuring nitrite levels, based on the formation of a chromophoric compound after the reaction with the Griess reagent (Basini *et al*. 2008).

SOD levels were measured employing a commercial assay kit (SOD Assay Kit, Sigma Chemical Co Lt, St. Louis, MO, USA). Scavenging non-enzymatic activity was evaluated determining the reducing ability of the plasma samples by a previously published technique (Ciccimarra *et al*. 2018).

The FRAP assay measures the change in absorbance at 620 nm due to the formation of a blue-colored Fe_2^+-tripyridyltriazine (TPTZ) compound from colourless oxidized Fe_3^+ form by the action of electron-donating antioxidants. Briefly, 20 µL of plasma were mixed with 20 µL of distilled water, and with 260 µL of FRAP reagent in each well of a 96-well plate. FRAP reagent was freshly prepared by mixing 25 mL acetate buffer (0.3 M; pH 3.6), 2.5 mL TPTZ (10 mM in 40 mM HCl), and 2.5 mL $FeCl_3 \cdot 6H_2O$ (20 mM). Aqueous solutions of known Fe_2^+- ($FeSO_4 \cdot 7H_2O$) concentration in the 100–1000 µM range were used for the calibration curve. The absorbance was recorded with Multilabel Counter Victor3 (Perkin Elmer, Boston, MA, USA) at 620 nm after a 30-min incubation at 37 °C (Huang *et al.* 2005).

5.4 – Statistical analysis

Statistical analysis was performed following using GraphPad Prism ver.7 software (GraphPad Software Inc., La Jolla, CA, USA). All data was tested for normality through the Kolmogorov-Smirnov test. Data with normal distribution were expressed as means ± standard deviation (SD), whereas data whose distribution was not normal were expressed as median value ± range. Mann-Whitney U Test was employed to evaluate the significance of the difference among groups of data, according to different HM, OS biomarker, sex, and age-class. Linear regression analysis and Pearson correlation coefficient analysis were used to assess the correlation between HM and OS biomarker levels observed.

Cap. 6 - Materials and methods concerning the study of OS in Blood of Domestic Chickens and Eurasian Magpies (*Pica pica*)

6.1 – Animals and sample collection

20 clinically healthy Tetra laying hens *(Gallus gallus domesticus*) from the same farm were enrolled in the study, divided into two different groups. Hens of Group 1 (G1, n = 10) were bred in an intensive indoor henhouse (8.7 hens/m^2), while those of Group 2 (G2, n = 10) were bred with a free-range system (0.6 hens/m^2). All hens were of the same age (37 weeks), genetic line, and fed the same diet. The mean productive levels were not significantly different between the two groups (6.44 eggs/hen per week in G1, and 6.36 in G2, P = 0.120).

On the same day (May 2019), a blood sample of each subject (1 ml) was taken from the right jugular vein, put in a heparinized vial, immediately placed in a refrigerated container, and carried to the laboratory. Each hen was then placed in a cardboard box for 1 hour, and its feces were collected for parasitological investigation. Blood collection was performed during routine health monitoring for the presence of infective bronchitis.

In parallel 18 magpies were captured (January-May 2019), in the proximity of the poultry farm area, in 4 capture sites located at a distance of less than 4 km from the farm. The capture area was an intensely cultivated zone, characterized by plenty of arboreal and forestry rows mainly consisting of *Populus spp*., *Junglas spp*., *Salix spp*., *Alnus spp*., *Morus spp*., at the transition between the rural and urban territory of Baganzola (Parma, Italy).

Magpies were captured by using no-kill traps (1 letterbox and 3 Larsen traps). The study was approved by the Emilia Romagna Regional Ethical Committee (ISPRA Prot. 8093/T-A31 of 02/21/19 and Region ER Det. 3751 of 01/03/19). Authorization included trapping of target species, capture modality (with lure birds), and all subsequent measurements, marking, and sampling procedures.

Identification of species, sex, and age was performed according to a previously published technique (Madge & Burn 1994), considering morphometric measurements (body weight, tarsal length, head and culmen). All captured magpies were considered clinically healthy and were evaluated for biometrical measurements, and marked with numbered tarsal open rings. A blood sample of each subject (1 ml) was collected, following the same procedure employed for hens. Birds were kept for the following 24 h in a single-subject cage with free access to food and water, to collect faecal

samples for parasitological investigation. After the conclusion of sampling operations all birds were released.

6.2 – Blood analyses

A 200 µl aliquot of each blood sample was used to perform the haematological assessment, according to the previously described technique (Polo *et al*. 1992). Microhematocrit was determined by centrifugation in a capillary tube; red blood cell count (RBC) and white blood cell count (WBC) were made using a haemocytometer using Natt & Herrick's stain; furthermore, a blood smear, stained with Haemacolor®, was assessed for differential white blood cell count. The remaining 800 µl of blood were used for plasma separation, and the samples were stored at -20° C until further analyses.

6.3 – OS biomarkers evaluation

d-ROMs and PAT levels were measured with a commercial spectrophotometric analyser (FRAS-5, H&D s.r.l., Parma, Italy), with a slight modification to manufacturer's instructions: 50 µl of plasma, instead of 10 µl, were used for d-ROMs determination. The d-ROMs level was expressed as UCARR units, 1 UCARR corresponding to 0.08 mg/dl H_2O_2, while PAT was expressed as UCOR units, 1 UCOR corresponding to 1.4 µmol/l ascorbic acid. The OS Index (OSI) was then calculated by the formula d-ROMs/PAT x 1000 and considered as an overall index of oxidative stress (Abuelo *et al*. 2013).

The plasmatic MDA levels, indicative of the extent of peroxidation phenomena affecting the phospholipids membrane, were determined following the previously illustrated protocol.

The plasmatic levels of UA were measured in consideration of the powerful antioxidant property of this compound which could influence PAT levels. The measurement was performed with a biochemical clinical automatic analyser (Fuji Dry-Chem 4000i, Fujifilm Europe GmbH, Düsseldorf, Germany).

All plasma samples were analyser for OS biomarkers and UA levels on the same day after a single thawing.

6.4 – Parasitological examination

The parasitological investigation for the presence of intestinal parasites was performed using the Mini-FLOTAC® technique, following the manufacturer's instructions. (Cringoli *et al*. 2017).

6.5 – Statistical analysis

Statistical evaluation of data was performed by using commercial software (GraphPad Prism®, ver. 6.0, GraphPad Inc., La Jolla, CA, USA), and Student's t-test was used to compare different data groups. Moreover, the level of correlation among data was evaluated by calculating the Pearson correlation coefficient (PCC). All data were expressed as mean ± SD, and a value of $P < 0.05$ was considered statistically significant.

Cap. 7 – materials and methods concerning the evaluation of redox and immune response following the administration of live attenuated *Mycoplasma gallisepticum* vaccination in backyard chicken

7.1 – Animals and sample collection

The work was developed at the same time of medical operations finalized to the normal management of the poultry farm and therefore falling within normal veterinary medical practices. The protocol of this study was submitted to the Committee for Animal Ethics of the University of Parma (approval number PROT. N. 23/CESA /2021).

The study involved a group of 12 subjects of Australorp hen (*Gallus gallus domesticus*), aged 23 weeks, including 2 males and 10 females, specimens kept extensively in a small amateur farm. The subjects were kept in a large brick hen house, with good hygienic and ventilated conditions (density in the henhouse 2.5 subjects/m^2), with the possibility to go out in a large 700 m^2 paddock with plenty of grass. The specimens were fed from the age of 6 weeks with commercial feed from laying hens first-period Ovaiole Sole Veg (Progeo Società Cooperativa Agricola - Via Asseverati, 1 Masone - 42122 Reggio Emilia - Italy) and watered with drinking water through a drop system, not other foods or supplements were provided.

At 10-week-old the group suffered from episodes of respiratory illness associated with MG infection, the episodes resolved and never recurred after treatment with doxycycline HCl in drinking water at 500mg/litre for 8 days, treatment associated with a thorough cleaning followed by a sanitization of the hen house, together all the elements contained in it, carried out by spraying a 1% (v/v) solution of sodium hypochlorite.

Given pathogen eradication plan from the group, at 22 weeks of age of the subjects, the vaccination was carried out by individual administration through intraocular route with live attenuated vaccine

Nobilis MG 6/85 fl 500d (MSD Animal Health), considering the vaccinal dose of $10^{6.9}$ – $10^{8.5}$ CFU of the live attenuated strain MG 6/85. At the moment of vaccination, Time 0 (T0), the animals were in optimal clinical state and the females had already started all spawning.

Simultaneously with the vaccination, a blood sample of each subject (2 ml) was taken from the right ulnar vein, put in a heparinized vial, immediately placed in a refrigerated container at 5°C, and carried to the laboratory. Each subject after the procedure has been individually marked through the application of a numbered tarsal ring, then placed in a cardboard box for 30 minutes, and its feces were collected for individual parasitological investigation.

The blood sampling from each specimen was subsequently repeated with the same procedure described above, specifically, it was repeated at 14 days (T1) and again at 21 days (T2) from T0. T0, T1, and T2 were defined considering the recommended time intervals for verifying the effectiveness of the vaccine response (Pakpinyo *et al*. 2013).

7.2 – Haematological analysis

The blood samples were managed following the method already adopted in the previous study phase, examining the levels of PCV, RBC, WBC, and the differential leukocyte count. The plasma obtained from each blood sample was divided into two aliquots, one intended for antibody titration for MG and one for measurement of OS biomarkers.

Antibody titration was performed with the commercial kit ID Screen® *Mycoplasma gallisepticum* Indirect (ID Innovative-Diagnostics, 310 rue Louis Pasteur – 34790 Grabels, France), a recombinant protein-based indirect ELISA, examination conducted following the procedure indicated by the manufacturer. Titles were expressed in S/P ratio, namely ratio between the optical density of the tested sample (S) and that of the positive control (P), with a positivity cut-off ≥0.5 (Feberwee *et al*. 2005).

7.3 – OS biomarkers analysis

Plasmatic biomarkers of OS were evaluated in all the magpies, to establish a possible correlation between HM levels and OS in this species. For this evaluation plasmatic OS biomarker levels were evaluated, considering the assay of MDA, d-ROMs, NO, O_2^-, SOD, and FRAP, following the same analytical method already described for the second study of the PhD project.

7.4 – Parasitological examination

The parasitological investigations were performed following the method adopted in the previous research study. The presence of intestinal parasites was investigated by performing the Mini-FLOTAC® technique, following the manufacturer's instructions. (Cringoli *et al*. 2017).

7.5 – Statistical elaboration

GraphPad Prism ver.7 software (GraphPad Software Inc., La Jolla, CA, USA) was performed for statistical elaboration. Kolmogorov-Smirnov test was employed for testing of distribution typology of obtained data group, and the result was expressed in means ± standard deviation for values with normal distribution than as median and range for values with not normal distribution. The evaluation of the significance of the difference among groups of data Mann-Whitney U-Test was employed for not normal distribution while unpaired t-test was performed for confronting of parametric data. Linear regression analysis and Pearson correlation coefficient analysis were used to assess the correlation between leucocytic level, S/P ratio, and OS biomarker levels observed.

RESULTS AND DISCUSSIONS

Cap. 8 - results and discussions concerning the study of Feathers Heavy Metal contamination in relation with OS state in Eurasian Magpies (*Pica pica*)

In the course of the study, 64 magpies were captured and sampled. 33 were females (17 young and 15 adult), and 31 were males (12 young and 19 adult).

From feathers samples collected were highlighted median HM concentrations respectively of Ni 0.68 mg/kg (0.18 – 2.27), Cd <LOD mg/kg (<LOD – 0.25), Pb 2.80g/kg (0.41 – 17.66), Hg 3.90 mg/kg (1.35 – 85.89) (Fig. 1). At the blood level the Hg content was evaluated highlighting values of 0.01 mg/kg (<LOD – 0.04). No correlation was found between the mercury levels found in the feathers and those observed in the blood (fig. 2). This finding goes against what was expected since the blood levels reflect recent exposure situations to Hg, very close to the time of sampling, while on the contrary, the levels present in the feathers are independent of any recent exposures but highlight exposure situations that occurred during the feather eruption and are related to the blood levels of Hg present during the eruption phase (Dauwe *et al.* 2003).

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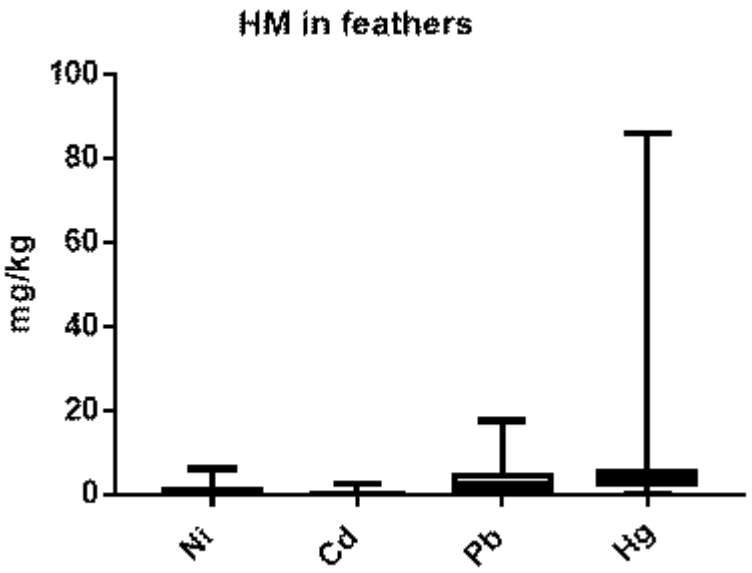
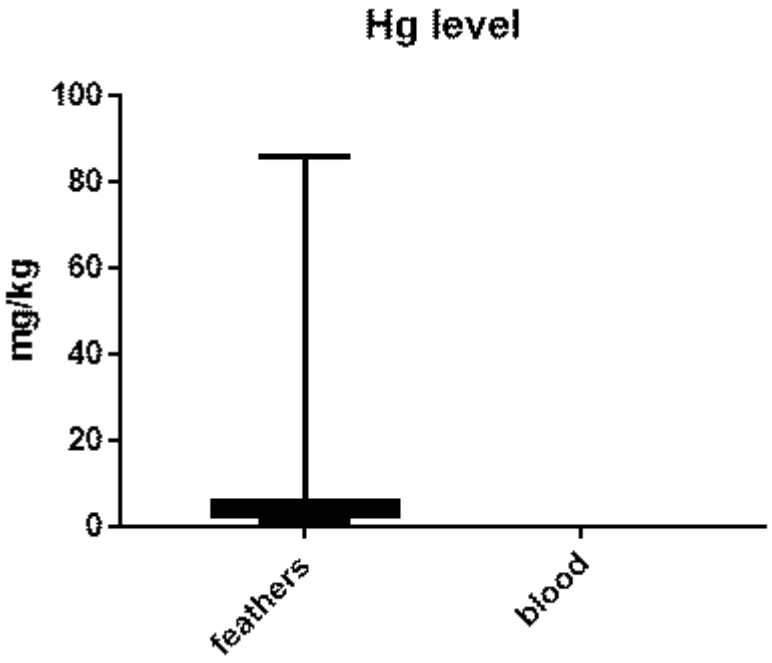


Figure 1: graphical representation of HM levels observed in feathers.

Figure 2: level of Hg in feather and blood.



Several previous works already exist that have taken into consideration the measurement of HM in feathers of wild continental sedentary birds as a strategy to evaluate environmental pollution as a bioindicator. None of these studies to our knowledge has considered *Pica pica* species. Two studies conducted in Malaysia in 2016 and successively in 2017 evaluated the levels of Pb, Ni, and Cd in the organs and feathers of *Corvus splendens*, belonging to the family of corvid as *Pica pica*. They highlighted a correlation for both metals between the concentrations found in the feathers and internal organs. In this case, however, showing respectively median values much higher (in particular ten times more hight for Pb and Ni) than those observed by us (Janaydeh *et al*. 2016; Janaydeh *et al.* 2017), it must be considered that the study in question was conducted in an area subject to strong industrial development, highly populated and urbanized, conditions that involve high HM emissions in the environment (Dayang *&* Che Fauziah 2013). In consideration of the ecological similarity of the two species in question (*P.pica* and C. *splendens*, with very similar ecological niches both opportunists omnivores) the high difference observed could be explained by the great difference in the different levels of contamination of the study areas, in detail of the degree of bioaccumulation of HM along the food chains characterizing the two areas.

In both the previous mentioned works the higher levels of Pb were found in the feathers and bone tissue, strengthening the hypothesis that the feathers analysis can be considered as a non-traumatic method on the sentinel species. It also suggests that it is a sensitive method for indirect monitoring of Pb environmental contamination level. The same applies to the highly detectable Ni in fine

tissues, including feathers. Therefore, we can hypothesize that the levels of Pb and Ni found in this study are indicative of the maximum levels of exposure with which the animals sampled have come into contact. Contrarywise, a high accumulation of Cd in the kidney and a low deposition in the feathers were observed, making the method less suitable for indirect monitoring.

Several other studies considered continental birds as sentinel species relative to HM levels, in a study of 2000, Dauwe *et al*. conducted in high polluted areas a study on *Cyanistes caeruleus* and *Parus major,* feathers (species belonging to the order of passerines such as *Pica pica*) sampled from the nest at the end of breading season, highlighting values of Cd and Ni in line with those observed by us, contrary to what was observed for Pb, which was more abundant when compared to the values we observed. The same situation relatively Cd, Ni and Pb levels emerges from the comparison with what is highlighted by the study of Dauwe *et al.* (2003) carry out from selected species of birds of prey (*Accipiter nisus, Athene nocta, Tyto alba, Stryx aluco*) from which, however, they emerged Hg level approximately 8 times lower than of those highlighted *P. pica* (Dauwe *et al.* 2003). Kim *&* Koo (2007) conducted a study in Korea on the feathers taken on two species of heron, highlighting in *Nycticorax nycticoraxin* and in *Ardea cinerea* highlighting in both species similar levels of Cd but values of Pb lower than those observed by us in *P.pica* (Dauwe *et al.* 2003).

In a study from 2012 (Kim *&* Koo 2007) feathers HM levels were measured considering pigeons (*Columba livia*) sampled in the highly urbanized and polluted area of Paris. Values observed for both metals (Pb, Cd) were much higher (5 times for Pb and more than 20 times for Cd) than those observed in *P. pica*.

Going to examine and compare the various works available, a high variability of the levels of the various HMs was observed. This is probably correlated with the levels of environmental contamination of the respective study areas, but certainly heavily influenced by the ecological niche of the different species subject to studies. In fact, apart from the two works on *C. splendens*, all the others have examined granivorous (Frantz *et al.* 2012), granivorous-insectivorous (Dauwe *et al.* 2000) or narrow carnivorous species (Dauwe *et al.* 2003) or ichtivorous carnivores (Kim *&* Koo 2007), which it makes very difficult to perform a comparison since the phenomenon of HM biomagnification is heavily influenced by the position in the food chain and therefore from the ecological niche (Janssen *et al.* 1993). Considering the plasmatic OS biomarker levels, MDA values of 23.64 mmol/L (± 14.41), NO 3.01 µM (1.48 – 11.53), SOD 8.28 U/ml (2.17 – 21.41), d-ROMs 0.06 UCAR (0.00 – 0.31), FRAP 293.10 µM (98.93 – 1727.00) and O_2^- of 0.21 mAbs (0.08 – 0.51) were

observed. No correlation was demonstrated between the levels of HM and OS biomarker taken into consideration. Therefore, we can say that the study of OS plasma biomarkers is not applicable as an indirect monitoring system of exposure levels to Cd, Ni, Pb, and Hg.

No significant differences were highlighted based on the gender distinction in the levels of any of the metals detected in the feather and blood samples (for Hg). For biomarkers of OS, a significant difference in MDA levels between males and females (p = 0.036) was found, with median values observed in females of 23.95 (1.10 - 81.60) mmol/L compared to those observed in males of 18.40 (1.30 - 53.00) mmol/L. (Fig. 3)

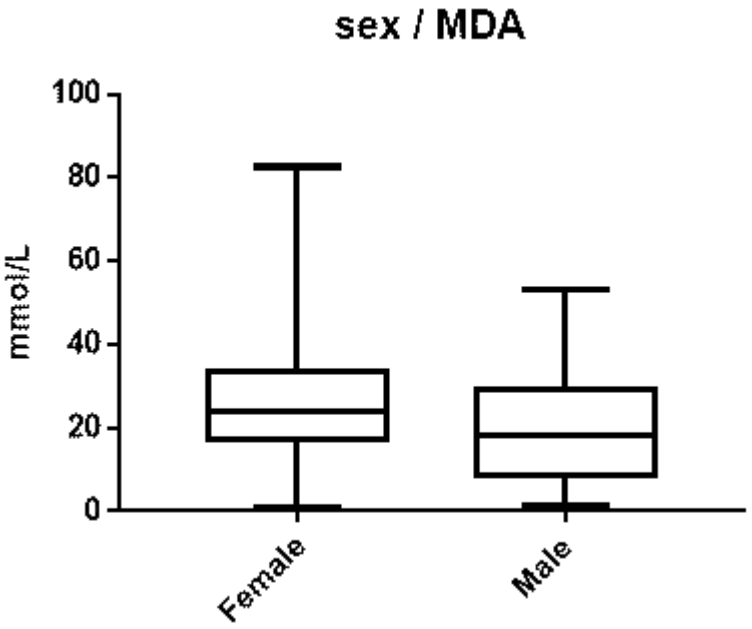


Figure 3: comparison of MDA levels considering gender subdivision.

Based on age class, a significant difference was highlighted for Pb levels (P = 0.003) and Ni levels (P = 0.047) in the feathers and Hg levels in blood (P = 0.0118), highlighting significantly lower levels in the young with median values respectively of Pb = 2.07 mg/kg (0.41 - 5.82), Ni = 0.54 mg/kg (0.18 – 2.27) and Hg = 0.01 (<LOD – 0.04), compared to those observed in adult specimens with Pb levels = 3.91 mg/kg (0.67 - 17.66), Ni = 0.54 (0.18 – 2.27) and Hg = 0.02(<LOD – 0.04), observation in accordance with what reported in previous studies (Giammarino *et al*. 2014; Naccari *et al*. 2009; Janaydeh *et al*. 2016). This is an aspect that can be explained by the greater needs of adults, due to the high energy expended for the reproductive activity that leads them to have higher food intakes than immature specimens. Consequently, a greater exposure to contaminants happens (Janaydeh *et al*. 2016). Significantly higher levels of Cd and Hg from feathers were found in young rather than in adults: for Cd (P = 0.0002) were found levels of 0.16 in youngs (0.12 – 0.20) while <LOD (<LOD – 0.25) in adults, for Hg (P = 0.0044) were found levels of 4.58 in youngs (1.54 – 85.89) and 3.26 (1.35

– 12.66) in adults. This is an aspect that could depend on the tendency of young *P.pica* subjects to exploit more anthropogenic food sources (garbage and waste), due to the lower ability to predate and search for natural foods comparing to more experienced animals (Madge *et al*. 1994). Hence, they will be exposed more to intake of materials polluted by these metals. In fact, following the application by the Italian Government of Regulation (EC) n° 1102/2008, which introduced a strong reduction in the use of mercury in production processes (historically major polluting sources) and a progressive reduction in civil use up to the banning in 2017 with regulation (EU) 2017/852, the sources of Hg pollution in the study area is probably primarily deriving from the disposal of contaminated materials for civil use, such as old batteries, old paints ad broken thermometers directly cast in the urban waste. If Hg were widespread in the food chain, Hg levels would be observed with more constant values within the population under examination. On the contrary, the high variability with extremely high peaks with absolutely non-parametric distribution could indicate point-like assumptions through the ingestion of materials at a high level of contamination (Driscoll *et al.* 2013).

Table 1: are reported median, range and p values regarding HM levels on the base of subdivision between young and adults specimens.

	Pb (mg/kg)	**Ni (mg/kg)**	**Cd (mg/kg)**	**Hg feathers (mg/kg)**	**Hg blood (mg/kg)**
Youngs	2.07 (0.41 – 5.82)	0.54 (0.18 – 2.27)	0.16 (0.12 – 0.20)	4.58 (1.54 – 85.89)	0.01 (<LOD – 0.04)
Adults	3.91 (0.67 – 17.66)	0.76 (0.23 – 2.26)	<LOD (<LOD – 0.25)	3.26 (1.35 – 12.66)	0.02 (<LOD – 0.04)
p values	p = 0.0036**	p = 0.047*	p = 0.0002**	p = 0.0044**	p = 0.0118*

Table 2. are reported median, range (not parametric distribution) or means and standard deviation (with parametric distribution) and p values regarding OS biomarkers levels on the base of subdivision between young and adults specimens.

	MDA (mmol/L)	**NO (µM)**	**SOD (U/ml)**	**d-ROMs (UCAR)**	**FRAP (µM)**	**WST (mAbs)**
Youngs	22.54 (±11.16)	3.28 (1.74 – 11.53)	8.04 (2.17 – 21.41)	0.06 (<LOD – 0.31)	282.50 (106.40 – 1727.00)	0.20 (0.08 – 0.47)
Adults	20.75 (±16.83)	2.98 (1.48 – 6.2)	5.82 (0.66 – 13.51)	0.07 (0.01 – 0.18)	303.60 (98.93 – 632.2)	0.21 (0.09 – 0.51)
p values	p = 0.5510	p = 0.2390	p = 0.2003	p = 0.8166	p = 0.6691	p = 0.8975

Considering statistically the relation between HMs levels, a significant positive correlation was observed between the levels observed in feathers of Pb and Ni ($r = 0.601$, $p < 0.001$).

OS biomarker showed a strong negative correlation between O_2^- and SOD levels ($r = -0.838$, $p < 0.001$).

Comparing the 8 sites examined, 4 sites concerned rural areas with 36 subjects sampled, in the specific Site 1 (n = 6), Site 2 (n = 14), Site 3 (n = 6) and Site 4 (n = 10) then 4 sites with 28 subjects belonging to urban areas, Site 5 (n = 3), Site 6 (n = 8), Site 7 (n = 11) and Site 8 (n = 6).

Considering the subdivision by urban area and rural area, a significant difference in the levels of mercury in the feathers was highlighted, detecting levels of Hg 4.05 mg/kg (1.35 – 12.66) in RZ then level of 2.99 mg/kg (1.54 – 85.99) in UZ, highlighting a significant difference in the observed levels ($p < 0.05$). No correlations were observed between the environmental conditions and the levels of other metals or with the values of OS with exception of FRAP with observed levels of 258.30 µM (98.93 – 630.20) in RZ then level of 320.90 µM (106.40 – 1727.00) in UZ.

In consideration of the comparison of the HMs values with the results reported in the bibliography, the values observed by us probably testify too low levels of HM pollution in general of all the areas involved in the study, or at least suggest the absence of constant sources of pollution, but more in the presence of events or point-like pollutant releases which are inconstant both in quantitative and temporal terms, in particular as regards Hg.

Table 3: are reported median, range and p values regarding HM levels on the base of subdivision for rural (RZ) or Urban (UZ) zone.

	Pb (mg/kg)	**Ni (mg/kg)**	**Cd (mg/kg)**	**Se (mg/kg)**	**Hg feathers (mg/kg)**	**Hg blood (mg/kg)**
RZ	2.60 (0.48 - 9.76)	0.66 (0.18 – 2.27)	<LOD (<LOD – 0.25)	1.10 (0.56 – 2.33)	4.05 (1.35 – 12.66)	0.01 (<LOD – 0.04)
UZ	2.89 (0.41 – 17.66)	0.74 (0.2 – 1.39)	<LOD (<LOD – 0.18)	0.97 (0.57 – 1.69)	2.99 (1.54 – 85.89)	0.01 (<LOD – 0.04)
p values	p = 0.7598	p = 0.6793	p = 0.8491	p = 0.0848	p = 0.0048*	p = 0.4983

Table 4: are reported median, range and p values regarding OS biomarkers levels on the base of subdivision for rural (RZ) or Urban (UZ) zone.

	MDA (mmol/L)	NO (µM)	SOD (U/ml)	d-ROMs (UCAR)	FRAP (µM)	WST (mAbs)
RZ	25.67 (±16.85)	3.01 (1.48 – 5.98)	8.40 (2.17 – 16.52)	0.07 (±0.11)	258.30 (98.93 – 632.20)	0.20 (0.09 – 0.47)
UZ	22.10 (±9.54)	3.00 (1.85 – 11.53)	8.11 (2.43 – 21.41)	0.08 (±0.07)	320.90 (106.40 – 1727.00)	0.23 (0.08 – 0.31)
p values	p = 0.3205	p = 0.5954	p = 0.6870	p = 0.2421	p = 0.0369*	p = 0.9949

Cap. 9 - results and discussions concerning the study of OS in Blood of Domestic Chickens and Eurasian Magpies (*Pica pica*)

In the 20 hens of the study, subdivided into the two groups, intensive G1(n: 10) and free-range G2 (n = 10), were observe d-ROMs values of 39.7 ± 16.4 and 19.0 ± 37.6 UCARR, while PAT levels were 2820.1 ± 420.4 and 3243.1 ± 427.7 UCOR, respectively (Fig 1). OSI resulted significantly higher in hens of G1 (14.7 ± 7.1) concerning those of G2 (5.6 ± 10.3), p = 0.04 (Fig. 4).

A significantly higher MDA concentration was detected in plasma samples of G1 hens (27.2 ± 10.4 nmol/ml) compared to G2 (8.2 ± 13.3 nmol/ml), p = 0.004 (Fig. 4). A strong positive correlation (PCC = 0.89) was found between OSI and MDA levels in hens, considering both experimental groups. By contrast, the results showed a negative correlation (PCC = -0.51) between PAT and MDA levels.

Mean haematological parameters, UA levels, and differential white blood cell count of hens are summarized in Table 5 and 6. Both PCV and RBC were significantly higher in hens of G2 (36.4 ± 3.2% and 2.7 ± 0.2 x 10^6 cells/dl respectively) with respect to G1 (30.1 ± 1.5% and 2.3 ± 0.3 x 10^6 cells/dl respectively), P = 0.006, and P = 0.0002. No significant differences were found in UA levels comparing hens of G1 and G2 groups, and no correlation was detected between UA and OS biomarkers or haematological parameters. A mild correlation (PCC = 0.31) was found between MDA level and WBC in hens of both groups. All hens resulted negative for intestinal parasitic infestations.

Figure 4: evaluation of OS biomarkers, considering d-ROMs, PAT, OS index, and MDA, in plasma of hens subdivided in G1 (intensive) and G2 (free-range). UCARR indicates Carratelli units, where 1 UCARR ¼ 0.08 mg/dL H_2O_2; UCOR, Cornelli units, where 1 UCOR = 1.4 lmol/L ascorbic acid. Data are expressed as mean 6 SD. * p <0.05, ** p <0.01.

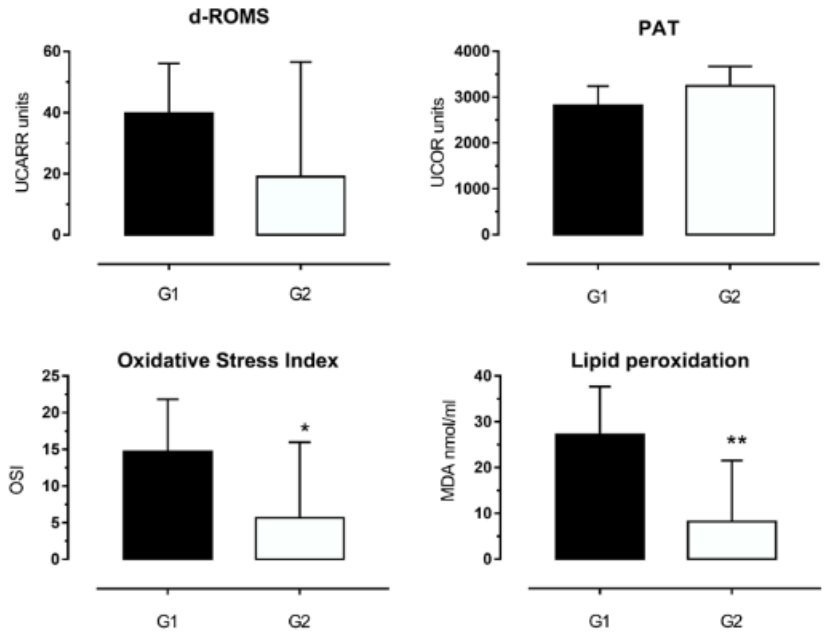


Table 5: haematological parameters and UA levels in hens bred with intensive (G1) or free-range (G2) methods. All data are expressed as mean ± SD. ***P* < 0.01 G1 *vs* G2.

	PCV ***(%)***	**WBC** ***(x 10^3 cells/dl)***	**RBC** ***(x 10^3 cells/dl)***	**Thrombocytes** ***(x 10^3/cells dl)***	**UA** ***(mg/dl)***
Group 1	30.8 ±1.5**	20.3 ± 17.5	2.3 ± 0.3**	39.7 ± 15.5	5.4 ± 1.8
Group 2	36.4 ± 3.8	10.8 ± 5.16	2.7 ± 0.2	23.1 ± 8.9	6.4 ± 2.3
Total	32.9 ± 4.0	16.1 ± 14.0	2.5 ± 0.3	32.3 ± 14.0	5.9 ± 2.0

Table 6: average values expressed in % relative differential leucocyte cell count detected in hens. All data are expressed as mean ± SD.

	Heterophils (%)	**Lymphocytes (%)**	**Monocytes (%)**	**Basophiles (%)**	**Eosinophils (%)**
Group 1	63.2 ±16.5	32.7 ± 14.2	1.7 ± 4.4	0.9 ± 1.8	1.6 ± 3.3
Group 2	60.0 ± 20.1	36.5 ± 21.2	1.6 ± 4.6	0.4 ± 1.1	1.3 ± 3.5
Total	61.8 ± 17.7	34.4 ± 17.2	1.7 ± 4.4	0. 6 ± 1.5	1.4 ± 3.3

Overall OSI values and lipid peroxidation levels in hens bred in high density and indoor conditions were significantly higher comparing to the ones that were bred with a free-range outdoor farming method. Even though the differences in d-ROMs and PAT values between G1 and G2 groups did not reach statistical significance, both a higher production of ROMs and a lower plasmatic antioxidant power in G1 hens rather than G2 hens might be hypothesized. As a matter of fact, a significantly higher d-ROMs/PAT ratio was calculated. Indeed, it was previously observed that exposure to stressful environmental conditions such as high stocking density (Simitzis *et al.* 2012), poor ventilation, and high dust levels, (Bottje *et al*. 1998) can increase ROMs production and OS in broiler chickens. In addition, since it was suggested that the antioxidant capacity in different subjects of the same species is mainly correlated to nutritional factors (Costantini *&* Dell'Omo 2006), the group of free-range hens in our study might have had access also to natural food sources such as grass and insects, potential sources of antioxidant compounds, thus ameliorating overall antioxidant defence.

Moreover, besides the finding of a significant increase of plasmatic MDA levels in G1 hens compared to G2 ones, a strong positive correlation between MDA levels and OSI or d-ROMs, and a negative correlation between MDA and PAT was detected in both groups, thus suggesting that OS biomarkers are indeed correlated to cell membrane phospholipids damage.

Even though there are already several studies on OS in poultry farms, it may be difficult to compare the results with the data obtained in our experiments, since most of these previous studies assessed the changes in OS following the exposure to stressors of various nature (such as thermal stress or experimental infections) or the administration of foods or supplements with antioxidant action. However, in these works, FRAP values, which are closely related to PAT values (PAT = FRAP/1.4), and MDA levels were found to be considerably lower than those observed in our study (Lin *et al.* 2008; Star *et al*. 2008). No previous data is available on d-ROMs values in hens.

Significantly lower RBC and PCV values in hens of G1 were observed concerning G2.A possible reason for this difference could be the damaging action by ROMs on erythrocyte membranes and haematopoiesis, since it has been previously observed that OS is responsible for haemolytic anaemia worsening (Fibach *&* Rachmilewitz 2008).

Magpies were 18 mature subjects (7 females and 11 males) of estimated age > 1 year. In magpies, the mean d-ROMs level resulted to be 17.5 ± 10.5 UCARR, while PAT was 2117.0 ± 178.8 UCOR (Table 7). The OSI value in magpies was 8.1 ± 4.6, while MDA level resulted to be 0.7 ± 1.6 nmol/ml (Table 7).

A mild positive correlation was found between OSI and MDA plasmatic concentration in magpies (PCC = 0.36); PAT and MDA levels resulted also positively correlated (PCC = 0.42). No significant difference was found among magpies according to sex, except for PAT value which resulted higher in males (2184.0 ± 168.5) compared to females (2012.0 ± 148.9), P = 0.04 (Table 7).

Table 7: Average values of OS biomarkers in magpies considering separately male and female subjects. All data are expressed as mean ± SD. *$P < 0.05$ Males *vs* Females.

	d-ROMs ***(UCARR)***	**PAT** ***(UCORR)***	**OSI** ***(1/1)***	**MDA** ***(nmol/ml)***
Males	17.4 ± 12.1	2184 ± 168.5	7.7 ± 5.0	0.2 ± 0.4
Females	17.7 ± 8.1	2012 ± 148.9 *	8.8 ± 4.2	1.5 ± 2.4
Total	17.5 ± 10.5	2117.0 ± 178.8	8.1 ± 4.6	0.7 ± 1.6

The data referred to haematological analysis, UA levels, and differential white blood cell count in magpies are summarized in Table 8. Male subjects showed significantly higher RBC values (3.8 ± 0.3 x 10^6 cells/dl) comparing to females (3.1 ± 0.5 x 10^6 cells/dl). Interestingly, in magpies with WBC above median value (20.4 x 10^3 cells/dl) d-ROMs and OSI were significantly higher (P = 0.03) compared to subjects with lower WBC (Fig 5). WBC resulted positively correlated to both OSI and MDA levels (PCC = 0.45 and 0.33, respectively) following the same trend as previous data.

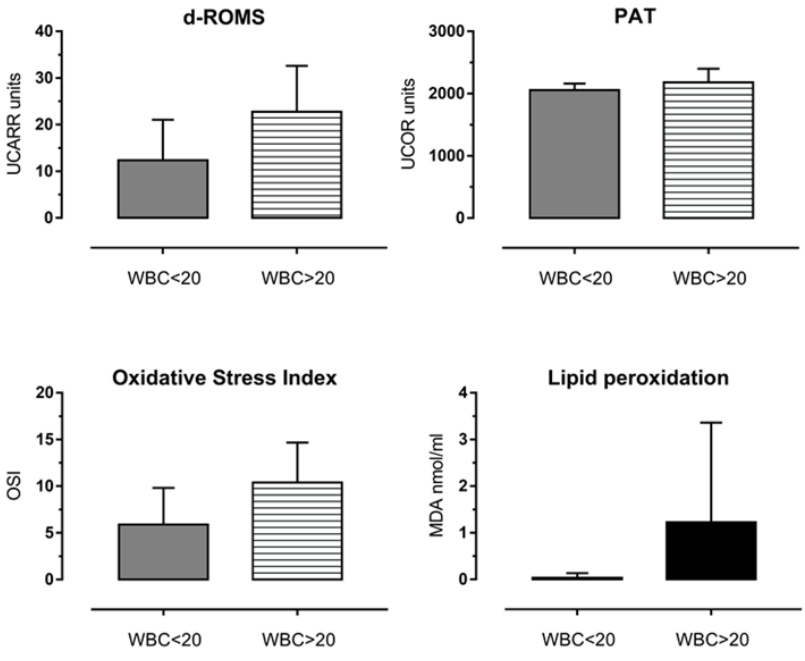


Figure 5: evaluation of OS biomarkers, considering d-ROMs, PAT, OS index, and MDA, in plasma of magpies, considering two group divided according to total white blood cell count (WBC) above or below the median value (MV; 20.43*10^3 cells/µL). UCARR indicates Carratelli units, where 1 UCARR ¼ 0.08 mg/dL H_2O_2; UCOR, Cornelli units, where 1 UCOR = 1.4 lmol/L ascorbic acid. Data are expressed as mean ± SD. Total WBC count < MV versus total WBC count > MV.

Table 8: average haematological values and means UA level detected in magpies considering separately male and female subjects. All data are expressed as mean ± SD. ** $P < 0.01$ Males *vs* Females.

	PCV *(%)*	WBC *(*10^3 cells /dl)*	RBC *(*10^6 cells / dl)*	Thrombocytes *(*10^3 cells / dl)*	UA *(mg/dl)*
Males	53.1 ± 10.1	14.6 ± 7.0	3.8 ± 0.3	10.8 ± 8.5	9.7 ± 1.6
Females	48.2 ± 2.3	31.3 ± 8.5	3.1 ± 0.5**	13.3 ± 6.3	9.9 ± 1.5
Total	50.1 ± 6.7	21.1 ± 11.2	3.4 ± 0.5	11.8 ± 7.6	9.8 ± 1.5

Table 9: Average values expressed in % relative differential leucocyte cell count detected in Magpies. All data are expressed as average values ± SD.

	Heterophils *(%)*	Lymphocytes *(%)*	Monocytes *(%)*	Basophiles *(%)*	Eosinophils *(%)*
Males	39.9 ± 21.6	57.1 ± 22.5	0.4 ± 1.0	0.2 ± 0.6	2.4 ± 2.5
Females	29.2 ± 8.9	63.1 ± 13.5	0.4 ± 1.1	0.9 ± 2.5	6.4 ± 6.5
Total	35.7 ± 18.2	59.4 ± 19.2	0.4 ± 1.0	0.5 ± 1.6	3.9 ± 4.8

No significant differences were found in magpies UA levels in different sexes. Also, no correlations were detected between UA and OS biomarkers or haematological parameters.

All magpies were diagnosed with a subclinical intestinal infestation of *Passerilepis* spp. cestodes. The stools of 8 magpies (5 females and 3 males) resulted also positive for coccidia of the genus *Isospora* (in all subjects < 30 oocysts/g of feces). However, the positivity for *Isospora* in the magpie group did not seem to be correlated with OS biomarkers or haematological findings.

To our knowledge, this is the first study regarding OS and haematological evaluation in magpies. Although a sub-group of magpies had an infestation by *Isospora*, no significant differences were observed in OS biomarkers or haematological parameters between positive and negative subjects. Indeed, the presence of subclinical coccidia infestations by *Isospora* spp. is reported both in domestic and wild bird species (Greiner *et al*. 2008). It is thus likely that in our study the parasitic infestation was not severe enough to alter haematological parameters or to induce an OS. Indeed, it was surprisingly observed that an anthelmintic treatment induced an increase in the levels of OS biomarkers in nestlings of birds of prey (Hanssen *et al.* 2013).

Significantly higher PAT levels were found in male magpies rather than females, just like what we observed for *Taeniopygia guttata*. In this species males showed higher levels of overall antioxidant defences concerning females (Alonso-Alvarez *et al*. 2004).

Sex seems to influence also haematological parameters in magpies since significantly higher RBC values were found in males. Once again, we can see the same trend observed in other avian species and could be explained by the erythropoietic action of androgens (Colin 2012). This result, however, should be further assessed also considering the influence of OS levels; a previous study (Alonso-Alvarez *et al*. 2007) suggested the existence of a negative correlation between testosterone levels and erythrocyte resistance against ROMs-induced damage.

We have found an interesting connection between OS biomarkers and WBC values. Indeed, in magpies with WBC above median value, significantly higher d-ROMs and OSI were detected, together with a moderate positive correlation between d-ROMs or OSI values and WBC. It is well known that immune activation with the consequent white blood cell proliferation and activation leads to an increase in the production of ROMs (Birden *et al.* 2012; Dalle Donne *et al*. 2006; Nguyen *et al*. 2017). Therefore, this could support, at least partially, these observations. No significant differences in the levels of MDA were however observed according to WBC levels, and only a weak positive correlation between MDA levels and WBC values was found. This is not surprising since the higher WBC values might probably still be within a physiological range, thus in healthy subjects the higher ROMs production due to white blood cell activity could be efficiently counteracted by the antioxidant system, preventing actual oxidative damage to cell membranes.

It is important to note that although the values of d-ROMs and PAT measured in the two avian species were not too dissimilar, much lower MDA values were detected in magpies then hens ($P < 0.0001$). Even though it is difficult to compare the data obtained in different bird species, and a great variability according to the species has been previously reported (Cohen *et al.* 2007; Salmon *et al*. 2018). The higher lipid peroxidation levels in hens could be partially due to the more stressful living conditions connected to captivity, compared to the natural environment available for magpies. Furthermore, antioxidant enzymes were not investigated, thus a difference between the two species in the role played by this important defensive system cannot be ruled out.

The plasmatic UA levels were also measured, given its antioxidant power (Cohen *et al.* 2007; Costantini 2011). In our experimental conditions, UA levels did not seem to influence OS biomarkers or haematological parameters, since in both species no correlation was found between levels of UA

and OS biomarker, and no significant differences were found among the various groups and subgroups examined.

Limitations to the present study could be having the relatively small number of the enrolled birds. Another limitation might be that PAT value takes into consideration only some antioxidant compounds, while an important role in the endogenous antioxidant system is played by enzymes like superoxide dismutase or glutathione peroxidase which are included. Further studies on a larger sample and a more thorough evaluation of the elements participating to oxidative/antioxidative balance will be needed. An additional limitation to the study is that only healthy subjects were assessed, and future experiments including pathological subjects should be performed.

Cap. 10 – results and discussions concerning the evaluation of redox and immune response following the administration of live attenuated *Mycoplasma gallisepticum* vaccination in backyard chicken

Across the three sampling sessions, T0 (vaccine administration), T1 (14 days after T0), and T2 (21 days after T0), no significant changes in PCV and RBC were found, keeping respectively in a mean range of 37.38 ± 7.23% and 2.83 ± 0.66 × 10^6 cells/µl of blood.

As regards the leukocyte count, a predictable progressive increase in mean leukocyte levels was observed (Fig.6), although no statistically significant variation in WBC levels has been demonstrated. What observed is indicative of leukocyte activation, expected phenomenon associated with an immune response. Considering that following the correct execution of a vaccination intervention, an immune activation follows which is associated with a replication and leucocyte differentiation to which the noted increase in WBC is attributable (Pakpinyo *et al.* 2013). All haematological data and antibody titles subdivided for sample sessions are reported in Table 10.

Across the three sampling sessions, no significant changes in terms of leukocyte formula were observed, except for monocyte percentage, which showed a significant decrease between T0 and T1 ($p = 0.0162$), followed by an increase between T1 and T2, bringing the levels of monocytes in T2 to close values observed in T0.

Considering the single cell types count (cells × 10^3/µl), no significant variations emerged between the levels highlighted across the three sampling sessions. Nevertheless, in particular concerning Lym and Het, some interesting variations were observed: a progressive increase in the average levels of Lym between T0, T1, and T2, unlike that observed for the Het, for which there was a decrease in the levels circulating between T0 and T1 followed by a return of levels close to those of T0 in T2, as

showed in figure 7 and 8. In figure -quale- are represented means and standard error for parameters with normal distribution than with median and interquartile range for not parametric distributions. This could be explained by analysing the phenomena of cell recruitment in the initial stages of the inflammatory process and immunity activation. Following the entry of an antigen into a tissue or, in this case following the interaction with conjunctival mucosa, tissue macrophages, and dendritic cells intervene primarily in the uptake and antigen processing, with promptly activation of the cytokine cascade, secreting interleukins (ILs) among which IL-1, the first activator of innate nonspecific immunity and mediator for the activation of T cells (Dinarello 2009; Kaneko *et al*. 2019). The result is immediate tissue recruitment of innate immunity cells, including neutrophils with an early transition from the blood compartment to the tissues attacked by the antigen (Dinarello 2009), explaining the decline in plasma levels observed strictly post-vaccination. At the same time, under the influence of tissue and macrophage, interleukins IL-1 and IL-6, T-helper 1 lymphocytes (Th1) produce IL-2, a mediator of a variety of functions critical to the progression through the specific immune response, responsible for rapid and important promotion of leucocytic clonal replication and recruitment (Weining *et al*. 1996; Muller 2001; Shi *&* Pamer 2011). The progressive leukocyte clonal replication ensues which could explain the observed progressive lymphocyte increase and the return of neutrophil levels in T2 to the levels observed in T0. Realistically the significant percentage decrease in Mon % observed in T1 could depend on the increase in absolute terms of Lym passing from T0 to T1, not accompanied by a simultaneous increase in Mon circulating, an increase in Mon highlighted in T2, thanks to which the levels % of Mon in T2 return to be close to those observed in T0.

Figure 6: graphic representation of the WBC levels trend (cell x 10^3/µl) between the different sampling sessions.

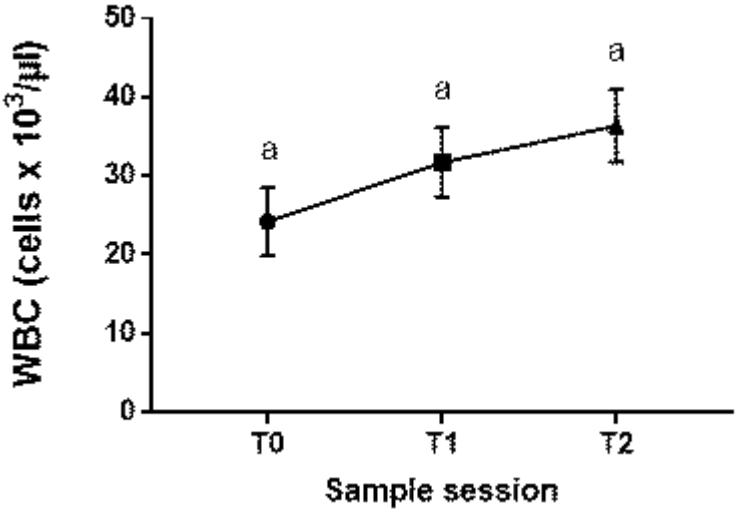
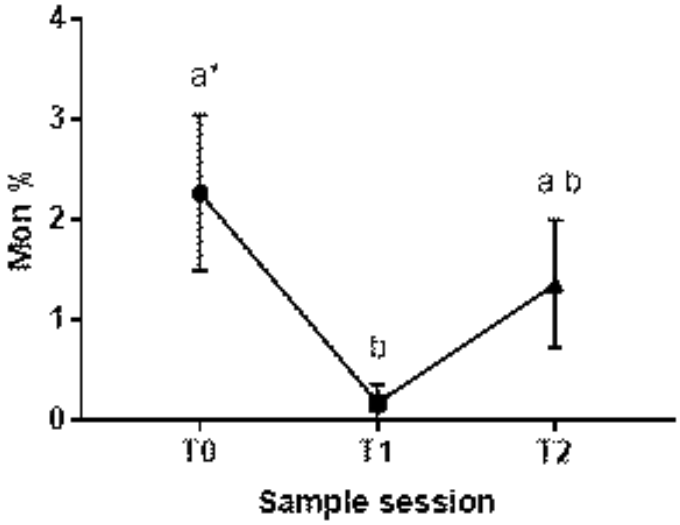


Figure 7: graphic representation of the trend of Mon levels (%) between the different sampling sessions; * p <0.05



Thrombocytes (Thro) also underwent significant changes, increasing between T0 and T1 (p = 0.0251) and then stabilizing and tending to a moderate decrease in T2, compared to the values observed in T1. What observed is compatible with what is known about avian thrombocytes, which are involved in innate immunity, as well as in the processes of thrombogenesis and hemostasis. They intervene in the very early stages of the immune response, in the identification and adhesion of not-self antigens as cells identify pathogen-associated molecular patterns (PAMPs) and in subsequent cytokines release preparatory to the activation of adaptative immune response (Scott *&* Owens 2008; Semple *et al*. 2011). Therefore, the observed increase could be directly associated with a clonal thrombocyte replication related to the exposure of the organism to vaccine antigens.

Figure 8: graphic representation of the trend of Lym levels (cells x 10^3/µl) between the different sampling sessions.

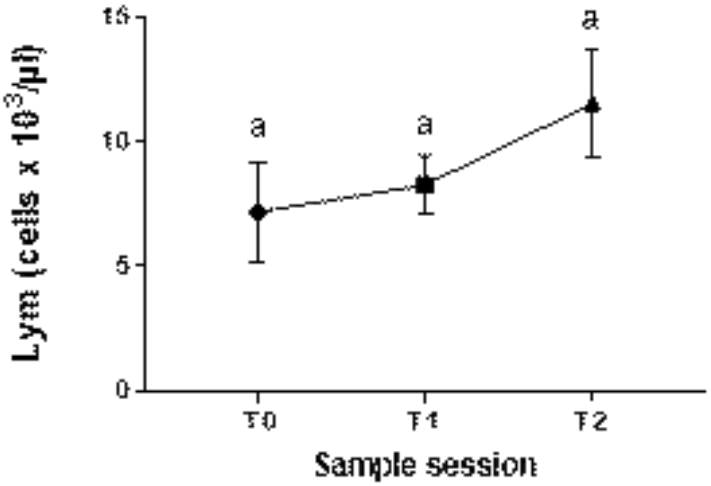


Figure 9: graphic representation of the trend of Het levels (cells x 10^3/µl) between the different sampling sessions.

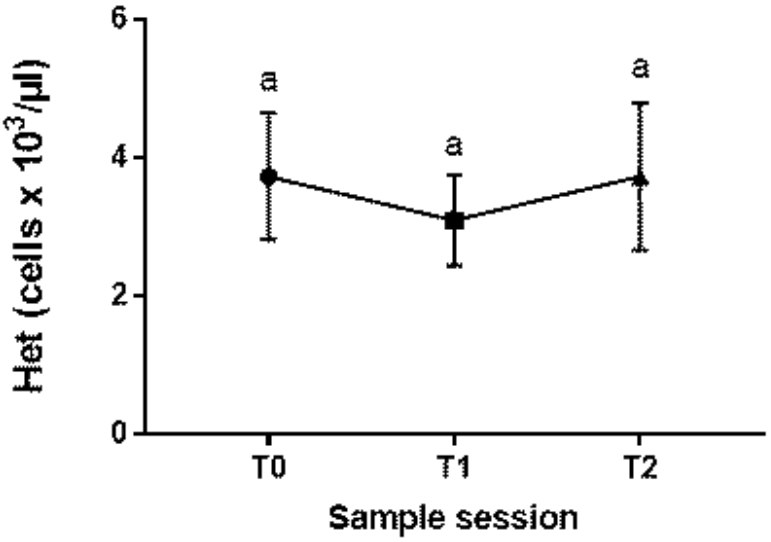


Figure 10: graphic representation of the trend of Thro levels (cells x 10^3/µl) between the different sampling sessions; * p <0.05.

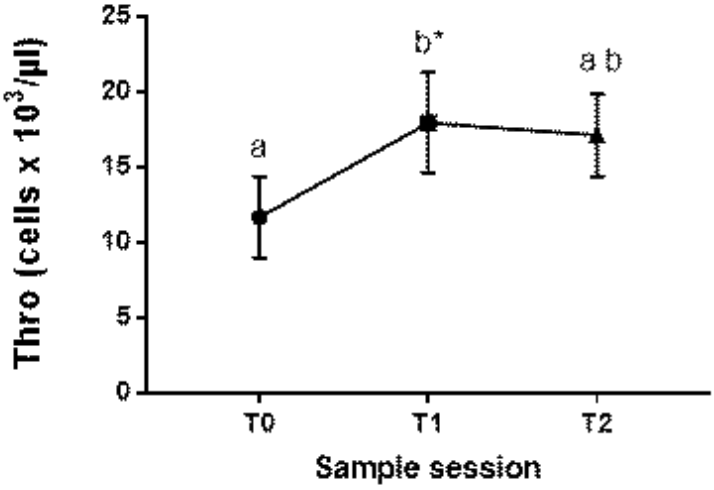


Table 10: the observed values for the single haematological parameters and antibody titers are indicated, divided for the different sampling sessions. Data are reported as means ± S.D.

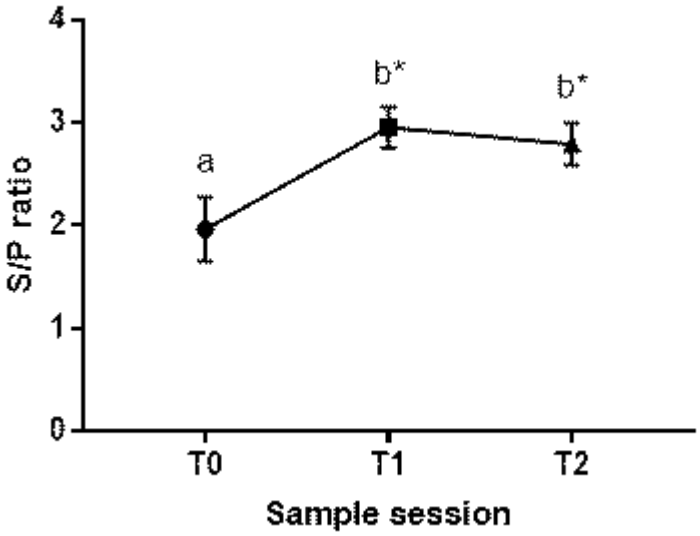
	T0	T1	T2
PCV (%)	36.61 ± 7.70	37.77 ± 7.95	37.75 ± 6.62
RBC (10^6/µl)	2.78 ± 0.38	2.87 ± 0.78	2.84 ± 0.79
WBC (10^3/µl)	12.21 ± 10.07	13.40 ± 5.25	18.93 ± 10.09
Het (%)	38.58 ± 16.54	32.75 ± 13.61	31.83 ± 9.04
Het (10^3/µl)	4.18 ± 3.16	3.47 ± 2.01	4.32 ± 3.52
Eus (%)	0.84 ± 1.59	2.20 ± 3.31	3.06 ± 2.98
Eus (10^3/µl)	0.21 ± 0.50	0.38 ± 0.68	0.59 ± 0.81
Bas (%)	0.64 ± 1.43	0.18 ± 0.40	0.59 ± 1.10
Bas (10^3/µl)	0.04 ± 0.10	0.02 ± 0.06	0.17 ± 0.40
Lym (%)	57.45 ± 14.49	64.79 ± 14.41	62.86 ± 10.22
Lym (10^3/µl)	7.61 ± 6.65	8.72 ± 3.85	12.00 ± 6.90
Mon (%)	2.53 ± 2.75	0.18 ± 0.60	1.66 ± 2.34
Mon (10^3/µl)	1.03 ± 1.22	0.03 ± 0.10	0.25 ± 0.29
Thrombocyte (10^3/µl)	11.97 ± 9.08	18.29 ± 11.09	17.42 ± 9.18
S/P ratio	1.96 ± 1.03	2.95 ± 0.65	2.79 ± 0.67

It can be considered that the means ± SD values observed for the different parameters found in the three sampling sessions returned within the normal limits available in the scientific literature (Carpenter & Marion 2018).

All subjects were found to be serologically positive for MG already in session T0, therefore all subjects already had a certain degree of immunity towards the pathogen for which they went to vaccinate. This is an expected event, considering that field infection exceeded in the months preceding the test. In this case the vaccination, given the pre-existing immunity, acted as a booster, reactivating the immune response acquired against MG.

As it should be expected, following the vaccination intervention there was a significant increase in the average antibody titer against MG, with $p = 0.0139$ between T0 and T1 followed by a moderate decrease of titer in the interval T1 – T2, however maintaining a significant difference between the average levels observed between T0 and T2 with $p = 0.0376$. Graphic representations of S/P trend are reported in figure 11.

Figure 11: graphic representation of the trend of S/P level between the different sampling sessions; *p <0.05.



After identification and consequent processing of already known non-self antigen by the first-line cells of the autonomous immune response (primarily macrophages, dendritic cells, and thrombocytes), the antigenic peptides are loaded onto the MHC II molecules and placed on the extracellular surface. At this point, under cytokines influence (firstly under IL-1) the T memory cell bind to the MCH II molecule/antigen and then induce the activation of the memory B cells (mainly promoted by IL-2). This determining a rapid cell replication and antibody production (Sharma 1991; Ahmed *&* Gray 1996), going to cause the rapid increase in antibody titer immediately following the vaccination intervention.

The decrease in antibody titer observed between T1 and T2 could be explained by the intervention of Lym regulators, through the stimulation of the release of IL-10 (with suppressive action) and the inhibition/subtraction of IL-2, with the progressive shutdown of the immune response (Sharma 1991; Min *et al*. 2001).

Examining OS biomarkers, a progressive - but not statistically significant - decrease in FRAP levels was observed from T0 to T2. FRAP trend has been graphically represented in figure 12. Contrarywise d-ROMs showed a progressive, although not statistically significant, increase from T0 to T2 (fig. 13). Concerning the MDA, extremely low levels were found in session T0, which underwent a rapid and significant increase in most of the vaccinated subjects (the 64% of subjects). This was already detectable in session T1 ($p=0.0258$) and evident and statistically significant for the interval T0-T2 ($p = 0.0004$).

In the time gap between T0 and T2, the hypothesis of immune activation can be advanced, explaining the increment of WBC value and S/P ratio following the vaccination intervention. It has been reported that the activation of the immune response involves an increase in the production and release of pro-oxidant factors (Costantini *&* Møller 2009), detectable by d-ROMs, which can lead to oxidative aggression and therefore peroxidation of membrane phospholipids (MDA is one of the products) (Zeb *&* Ullah 2016). Consequently, an inevitable consumption of antioxidant factors verifies, with particular reference to non-enzymatic molecular compounds of food origin such as ascorbic acid, among the elements measured by FRAP assay, particularly evident depletion when dietary intake is not sufficient to compensate for the loss (Whitehead *&* Keller 2003; Khan *et al*. 2012). Therefore, based on what has been observed, we can hypothesize that following the vaccination intervention an immune activation takes place, followed by a progressive release of pro-oxidant factors. Indeed, we registered an increase in d-ROMs, responsible for an oxidative attack on cell membranes demonstrated by the significant increase in MDA levels in the T0-T2 interval, and a concomitant and gradual depletion of antioxidant factors, demonstrated by the decrease in FRAP, probably consumed in scavenging processes.

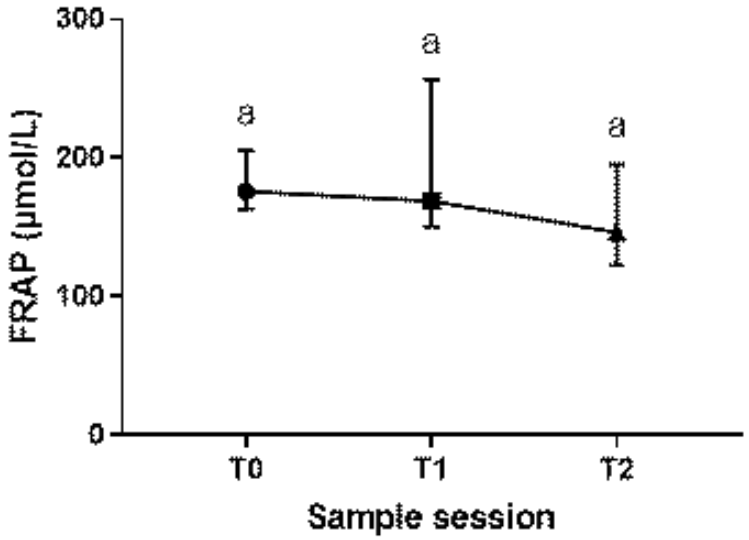


Figure 12: Graphic representation of the FRAP trend level between the different sampling sessions; median values are considered, for not parametric distribution.

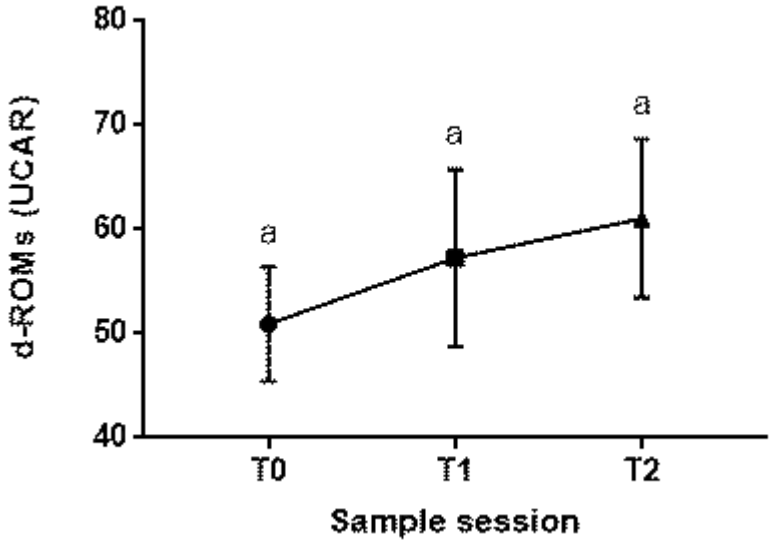


Figure 13: Graphic representation of the d-ROMs trend level between the different sampling sessions

Considering NO an early non-significant increase in the T0-T1 interval was observed. It was characterized by high variability of responses, followed by an evident, but not statistically significant, decrease in the T1-T2 interval. It is conceivable that the early, but slight, increase in NO levels is connected to the activation of tissue phagocytic cells (primarily macrophages), which, following antigenic stimulation and under the influence of cytokines (IL-1, IL-2), is responsible for the production and release of NO. NO indeed is a reactive compound with high oxidative potential, playing the role of cellular transmitter, in particular with the capacity of promoting leukocyte recruitment (Weining *et al*. 1996). The drop in levels in the T1-T2 interval could be connected to the intervention of Lym T regulators (Min *et al.* 2001), which through the release of IL-10 may be involved in the progressive decrease in immune activation with the consequent decrease in NO.

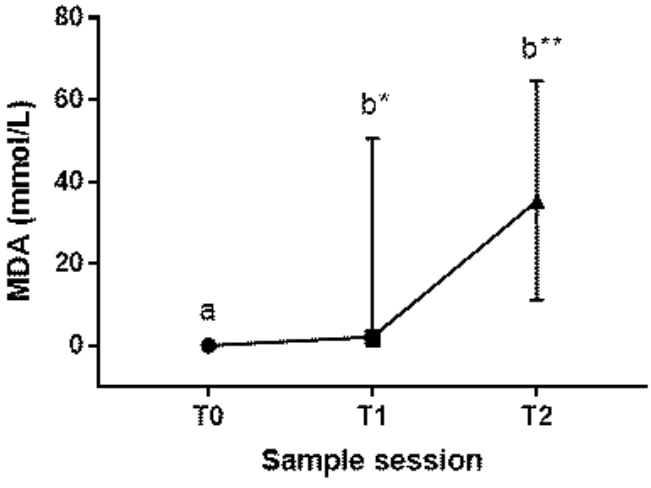
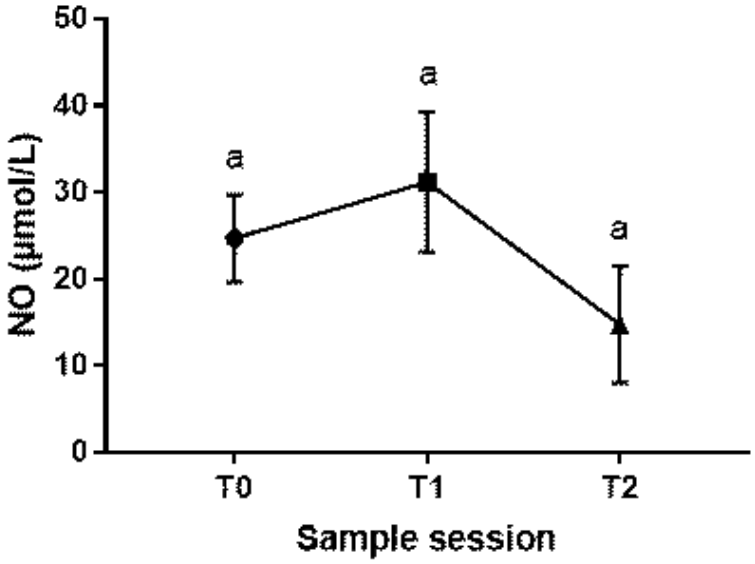


Figure 14: graphic representation of the MDA trend level between the different sampling sessions; median values are considered, for no parametric distribution; * p <0.05; ** p <0.01.

Figure 15: graphic representation of the NO trend level between the different sampling sessions.



Median and range for O_2^- levels (non-parametric distribution) remained almost constant in the T0-T1 interval, to subsequently undergo a moderate decrease in the T1-T2 interval (Fig. 16).

The mean levels of SOD (Fig. 17) undergo a very limited increase in the T0-T1 interval, while presenting a rapid increase after T1, going to determine a significant increase considering the interval T0 - T2 ($p=0.0472$). Superoxide is a reactive compound produced firstly following antigenic activation of PAMPs phagocytes, including neutrophils and macrophages (Scott *&* Owens 2008; Semple *et al*. 2011), to degrade the pathogen (Segal 2006). In this case the release is greater when the antigenic stimulation is massive and prolonged, such as in the case of chronic infection (Scott *&* Owens 2008).

In the present research, the vaccinal antigenic stimulus was probably insufficient to induce a relevant O_2^- production. Contrarywise following the immune activation, an increase in SOD levels is achieved. This becomes particularly evident and significant in the late phase, therefore between T1 and T2, which is accompanied by a reduction in O_2^-. This trend is in line with what is known, namely that the production and release of SOD, with direct action of degradation of the O_2^-, plays a fundamental role in the terminal phases of the benign inflammatory process. It interrupts the oxidative chain reaction, therefore limiting oxidative tissue damage, and limiting the progression of inflammation (Yasui *&* Baba 2006).

Figure 16: graphic representation of the superoxide trend level between the different sampling sessions. Median values are considered, for no parametric distribution.

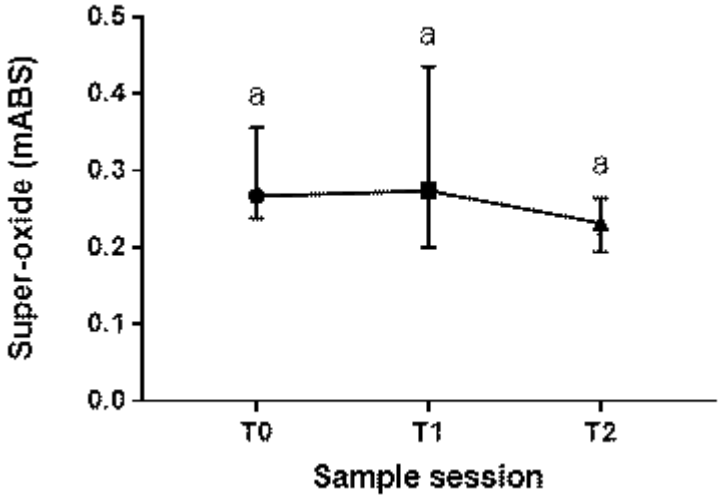
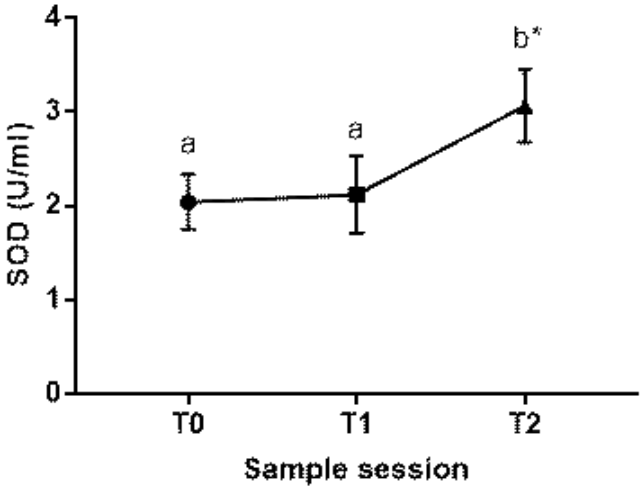


Figure 17: Graphic representation of the trend of SOD level between the different sampling sessions; *p <0.05.



Data related to OS biomarker subdivided for sample session are reported in table 11 for d-ROMs, NO, and SOD, considering means, and standard deviation (parametric distribution) and in table 12, represented as median ± range (non-parametric distribution).

Table 11: means and SD highlighted about d-ROMs, NO and SOD consider the different sample session.

	T0	T1	T2
d-ROMs (UCAR)	50.88 ± 18.19	57.20 ± 28.21	61.03 ± 25.16
NO (µmol/L)	24.69 ± 16.71	31.20 ± 26.73	14.74 ± 22.34
SOD (U/ml)	2.25 ± 0.72	13.40 ± 5.25	18.93 ± 10.09

Table 12: median and range highlighted about MDA, O_2^- and FRAP consider the different sample session.

	T0	T1	T2
MDA (mmol/L)	< LOD (<LOD – 4.00)	2.10 (<LOD– 172.60)	35.27 (<LOD – 90.20)
O_2^- (mABS)	0.27 (0.22 – 0.64)	0.27 (0.17 – 0.65)	0.23 (0.18 – 0.64)
FRAP (µmol/L)	175.99 (122.29 – 350.87)	169.07 (102.65 – 274.40)	146.58 (97.14 – 231.71)

For OS biomarkers levels in *G. gallus,* the comparable data available in the literature are unfortunately limited. The data available is often hardly comparable for the great heterogeneity of analytical methods employed and of biological substrates examined (Altan *et al*. 2003; Biazus *et al.* 2017). Moreover, attempting a comparison with findings obtained on other bird species, even starting from similar analytical methods and biological matrix, the comparison remains difficult for the great level of interspecies variability (Cohen *et al*. 2007; Salmon *et al*. 2018).

However, in two study of the PhD project research, we measured plasmatic levels of d-ROMs, PAT (PAT=FRAP/1,4) and MDA in two groups of laying hens maintained with two different breeding modality, intensive indoor (G1) and extensive outdoor (G2) (Iemmi *et al.* 2021). The levels of mean d-ROMs observed in the present study are in line although moderately higher than those observed in the previous study, specifically about 1.4 times higher than those detected for the G1 group and about 2.9 times compared to the average levels of G2. The mean FRAP values obtained in the present study were found to be markedly lower than those found previously: approximately 21 times lower than those observed for G1 and 24 times lower than those observed for G2. Contrarywise, they were found to be more in line, remaining in the order of 2.5 times the reported values, with the levels found in previous publications (Lin *et al.* 2008; Star *et al*. 2008). In this case, the high discrepancy could depend on the different methods used in the two studies, hence the importance of defining a standard method for the determination of OS biomarkers for diagnostic and clinical purposes.

The MDA levels we observed in T1 and T2 were in the same order of magnitude as previous works have found, as well as the ones observed in the control group of Lin *et al*. (2004). Therefore, we can assume that those are resulting from an activation of the immune response. Probably the group involved in this study before vaccination was subject to minimal levels of cell membranes oxidative damage, evidently smaller than that of the animals involved in the previous studies.

As for the SOD levels detected, they are much lower than those highlighted by previous works in the same species and biological matrix, resulting about 5 times lower than those observed by Lin *et al.* (2008) and approximately 2 times lower than those observed in Lin *et al*. (2004).The observed NO values are more or less stackable to those highlighted in the control group of the study by Zhao *et al* (2009) and to those described in Zhao *et all* (2009b).

Regarding the superoxide anion, no data useful for a comparison (obtained by *G. gallus* using plasma as a matrix) was found in the literature, therefore.

We then evaluated the degree of correlation between the performance of the various parameters examined so far, considering the two main indicators of the immune response state: the WBC level and the S/P ratio values, as the first base of correlation confront. The correlation levels are shown below in tables 13, 14, 15, 16, and 17: positive values are indicative of positive correlation (written in black) vice versa for negative values (written in red), values with a green background are indicative of strong correlation, orange for moderate correlation, white if no correlation was found.

Table 13: Pearson correlations by sampling session taking in reference the WBC values; leucites component are intended in the correlation calculation as cell x 10^3/µl of blood; positive values are indicative of positive correlation (written in black) vice versa for negative values (written in red), values with green background are indicative of strong correlation, orange for moderate correlation, white if no correlated.

Correlation level	Het	Lym	Mon	Eus	Bas	Trom	S/P	FRAP	d-ROMs	MDA	NO	O_2^-	SOD
WBC T0	0,938	0,986	0,439	0,745	-0,271	0,134	-0,171	-0,079	-0,310	-0,323	-0,269	0,002	0,479
WBC T1	0,512	0,889	0,253	0,546	0,014	0,564	0,184	-0,131	-0,306	-0,380	-0,128	-0,165	0,382
WBC T2	0,383	0,966	-0,287	0,049	0,528	0,257	-0,025	-0,140	0,244	-0,063	0,401	0,349	0,000

As expected, there was a positive correlation between WBC and the number of leukocyte components, firstly considering lymphocytes, eosinophils, and heterophile. In particular, we found that the levels of Het and Eus are highly correlated to the levels of WBC at T0before the immune stimulus occurred. Afterward they gradually lose correlation following vaccination in T1 and T2, while the levels of Lym remain constantly strongly correlated to the WBC, testifying, in this case, a predominantly lymphocytic response (Weining *et al*. 1996).

WBC and OS biomarkers in session T0 and T1 showed a moderate negative correlation between WBC and d-ROMs (r = -0.310; r = -0.306) and MDA (r = -0.323; r = -0.380) and positive with SOD (r = 0.479; r = 0.382), while in session T2 a moderate positive correlation with NO (r = 0.401) and O_2^- levels (r = 0.349).

Table 14: Correlations by sampling session with reference to S/P values; positive values are indicative of positive correlation (written in black) vice versa for negative values (written in red), values with green background are indicative of strong correlation, orange for moderate correlation, white if no correlated.

	WBC	Het	Lym	Mon	Eus	Bas	Trom	FRAP	d-ROMs	MDA	NO	O_2^-	SOD
S/P T0	-0,171	-0,276	-0,106	-0,368	-0,031	-0,240	-0,040	-0,225	0,721	0,031	0,209	0,464	-0,144
S/P T1	0,184	-0,014	0,153	0,432	0,068	-0,270	-0,382	0,073	-0,041	-0,200	-0,055	-0,203	0,533
S/P T2	-0,025	0,091	0,005	-0,207	0,494	-0,338	-0,279	0,239	0,339	-0,244	0,272	0,348	-0,386

Taking as a comparison reference the S/P values, no correlation was observed concerning the level of WBC, heterophile, lymphocyte, FRAP, MDA, and NO, while moderate but discordant correlations emerged compared to the levels of circulating monocytes, eosinophils, basophils, and thrombocytes. On the contrary in session T0 a strong positive correlation (r = 0.721) between S/P and d-ROMs was observed. A positive correlation was also detected in session T2 (r = 0.339), but not highlighted in session T1.The same trend was observed with O_2^-, with r = 0.464 in session T0 and r = 0.348 in session T1. Since in T0 the subjects had not yet undergone the vaccine infection, the S/P value depended exclusively on the stimulation linked to the field infection. Higher S/P in T0 could be associated either with a more pronounced and protracted individual immune response, linked to the subject's metabolic characteristics, or with a depletion of the most recent field infection, both scenarios justifying higher levels of oxidizing factors (indicated by d-ROMs and O_2^-) deriving from immune activation (Costantini & Møller 2009) and/or from the metabolic activity of the pathogen (Nascimento *et al.* 2005). The positive correlation observed in T2, however, reinforces the hypothesis that a more pronounced and especially more prolonged immune activation of the humoral type leads to greater releases and consequently to a progressive accumulation of oxidizing factors.

In addition to the correlation between the levels highlighted for a single sampling session, we went to evaluate the correlation between the variation (Δ) detected for each interval between the tree session (interval T0-T1, T1-T2, and T0-T2) of the two reference parameters (WBC and S/P), with the variation observed for the principal leukocyte component (Het and Lym count) and OS biomarkers.

Variation values are obtained through the calculation of the difference $\Delta WBC_{TxTy} = WBC_{Ty} - WBC_{Tx}$ and applying the same formula to other parameters. The variation of Mon, Eus and Bas levels aren't reported as we encountered no influent variations.

Table 15: Correlations by sampling session T0, T1 and T2, considering as reference WBC variation values (ΔWBC_{TxTy}); positive values are indicative of positive correlation (written in black) vice versa for negative values (written in red), values with green background are indicative of strong correlation, orange for moderate correlation, white if no correlated.

	ΔHet	ΔLym	ΔS/P	ΔFRAP	Δd-ROMs	ΔMDA	ΔNO	ΔO_2^-	ΔSOD
ΔWBC_{T0T1}	0,639	0,957	-0,233	-0,490	-0,526	-0,186	-0,571	-0,524	0,572
ΔWBC_{T1T2}	-0,099	0,956	0,329	-0,470	-0,351	-0,410	-0,473	-0,537	0,623
ΔWBC_{T0T2}	-0,237	0,964	-0,362	-0,031	0,416	0,171	0,049	0,167	0,008

Values of ΔWBC presented a strong positive correlation in all three intervals (always r> 0.956) with the trend of circulating Lym, as well as in T0-T1 a moderate positive correlation (r = 0.639) present with circulating heterophile. Therefore, following antigenic exposure, the observed leukocyte increase (ΔWBC) could be driven by an initial mixed leukocyte response, partly non-specific (Het) and largely specific (Lym), and then could be pushed in the medium/long term to a prevalent lymphocytic response, in line with what known on the topic (Weining *et al.* 1996).

Starting from the absence of correlation between WBC and S/P, by examining the values of ΔWBC and ΔS/P, poor and discordant correlation levels were observed, highlighting the absence of correlation in the trend of the two parameters in the T0-T1 interval to then become moderately positively correlated in T1-T2 (r = 0.329), that is, despite determining a negative correlation in the general period T0-T2 (r = -0.362). In a previous work done on *G. gallus* it was found that, following an important antigenic stimulus (in that case experimental infection with chicken Paramixovirus-1), an increase in both WBC and antibody titers takes place. Unfortunately, the correlation between the two parameters wasn't taken into consideration (Anunciado *et. al* 2007). In the present study, the absence of correlation in the first period T0-T1 could be justified by the fact that in the first phase immediately following the immune activation prevails a non-specific immune response, independent of antibody synthesis, progressively flanked by the specific one, in this case going to determine in a more advanced phase (T1-T2) a humoral response positively correlated with the leukocyte increase (Weining *et al*. 1996; Hasselquist & Nilsson 2012). The negative correlation between the values of ΔWBC_{T0T2} and $\Delta S/P_{T0T2}$ highlights that a marked leukocyte response both in terms of quantity and duration is counterproductive for antibody immunity.

Examining the relation of ΔWBC with OS biomarkers variations, there was a negative correlation with the values of ΔFRAP, Δd-ROMs, ΔNO, and ΔO_2^- in the intervals T0-T1 and T1-T2, negative with the ΔMDA for the T1-T2 interval, but positive with the ΔSOD in T0-T1 and T1-T2.

The non-enzymatic antioxidant component tends to be attacked by oxidative phenomena inevitably associated with immune activation (Costantini & Møller 2009) and this explains the negative correlation with ΔFRAP, but its progressive depletion is probably effectively compensated by the increase in SOD synthesis, though we cannot exclude the involvement of other elements such as Glutathione peroxidase (GSH-Px), glutathione reductase (GRD), and glutathione (GSH), (Gautam *et al.* 2010), following lymphocyte activation (leukocyte component preponderant), while keeping oxidative phenomena under control, which would explain the negative correlation with Δd-ROMs, ΔNO and ΔO_2^- and ΔMDA.

Table 16: Correlations by sampling session considering ΔS/P value variation. positive values are indicative of positive correlation (written in black) vice versa for negative values (written in red), values with green background are indicative of strong correlation, orange for moderate correlation, white if no correlated.

	ΔWBC	**ΔLym**	**ΔHet**	**ΔFRAP**	**Δd-ROMs**	**ΔMDA**	**ΔNO**	**ΔO_2^-**	**ΔSOD**
$\Delta S/P_{T0T1}$	-0.233	-0.153	-0.356	-0.363	-0.082	0.204	-0.200	-0.150	0.260
$\Delta S/P_{T1T2}$	0.329	0.318	0.042	-0.152	-0.163	0.160	0.024	0.128	-0.009
$\Delta S/P_{T0T2}$	-0.362	-0.377	-0.087	-0.095	0.278	-0.295	-0.329	-0.526	0.659

As predictable, the same relation already observed between ΔS/P and ΔWBC appears between ΔS/P and ΔLym; since in the specific case the WBC is strongly determined by the entity of Lym, this result supports the hypothesis that the circulant leukocyte and lymphocyte increase does not lead to greater antibody production.

For the T0-T1 interval a moderate negative correlation with ΔHet (r = -0.363), explained by the prevalence of the nonspecific immune response to the detriment of the humoral one in the early phase of the immune response (T0-T1).

Considering the ΔFRAP, only a moderate negative correlation (r = -0.363) with ΔS/P in the T0-T1 interval was observed, probably linked to the consumption of not enzymatic antioxidant factors linked to immune activation, as already observed, even if more markedly, considering the ΔWBC. No correlation has been found between ΔMDA and ΔS/P.

Moreover, referring to ΔS/P we have highlighted for the interval T0-T2 a negative correlation with ΔNO (r=-0.329) and in particular with ΔO_2^- (r = -0.526), while a positive correlation with ΔSOD (r=0.659), which could be explained by the fact that the increased oxidative pressure leads to an inhibition of antibody production, but this does not happen if the immune response is directed

towards the increase of antioxidant enzymes (SOD), with consequent reduction of the oxidants compounds and consequent increase in antibody synthesis.

After comparing the values of the various parameters for each sampling session and the variations considering the intervals between the samples, we finally examined the variations of WBC and S/P for the intervals T0-T1, T1-T2, and T0-T2, regarding the levels of OS biomarkers highlighted at the beginning and the end of the interval.

Starting from the FRAP, as regards the values of ΔWBC for the various intervals, no significant correlations were highlighted, on the contrary, a moderate positive correlation (r=0.396) was highlighted between $FRAP_{T0}$ and S/P_{T0T1}. This might indicate that, following an antigenic stimulus, higher levels of antioxidants in the plasma favour a greater antibody response at least in the very short period (in the first 7 days from the stimulus).

Interestingly, d-ROMs are constantly positively correlated with the variation of WBC (as shown in Table 8), in particular highlighting that high leukocyte proliferation could be associated with final high levels of d-ROMs and therefore free radicals; on the contrary, d-ROMs negatively correlated with the variation of S/P specifically with high $d\text{-}ROMs_{T0}$ there is associated a lower release of antibody for $\Delta S/P_{T0T1}$ (r =-0.488) and $\Delta S/P_{T0T2}$ (r = -0.570).

Table 17. Study of the correlation between levels of d-ROMs for the single sampling sessions and the variation of WBC levels between the different sessions. Positive values are indicative of positive correlation (written in black) vice versa for negative values (written in red), values with orange background are indicative of moderate correlation, white if no correlated.

ΔWBC_{T0T1}		ΔWBC_{T1T2}		ΔWBC_{T0T2}	
$d\text{-}ROMs_{T0}$	$d\text{-}ROMs_{T1}$	$d\text{-}ROMs_{T1}$	$d\text{-}ROMs_{T2}$	$d\text{-}ROMs_{T0}$	$d\text{-}ROMs_{T2}$
0.400	-0.134	0.643	0.446	0.386	0.604

Considering the MDA values, no significant correlations were highlighted, while concerning the NO and O_2^- levels, little-significant/inconsistent correlations were highlighted compared to the variations of WBC and S/P, as for SOD levels referred to ΔWBC, while a positive correlation between $\Delta S/P_{T0T2}$ and SOD_{T2} (r = 0.512) emerged, reinforcing the hypothesis that SOD production is closely related to a high antibody response (Gautam *et al.* 2010).

The study then highlighted some critical issues, particularly in the small sample size (12 specimens), the lack of a control group, the time taken into consideration (only 21 days), and the fact that a group of subjects was considered already presenting an immune memory towards the antigen used,

problems justified by the fact that the present work is not associated with an experimental project but rather with a clinical health intervention. For these reasons, the present study should be considered a preliminary work based on which subsequent works on experimental groups could be set up.

Cap. 11 – Conclusion

We conducted an indirect monitoring of the environmental contamination level by THMs based on sentinel species observation. Considering first and foremost the levels of Pb and Hg in *Pica pica* feathers, this appears to be a sensitive method, since measurable levels were highlighted in most of the samples examined, despite the presumed low levels of dispersion of THMs in the environment under study (Harmens & Norris 2008).

To obtain a greater sensitivity in the evaluation of Ni and Cd, it would be necessary to widen the range of the samples examined, extending the study on the renal and hepatic tissue. this of course would require sacrificing the sentinel specimens sampled, a situation which is however feasible if the sentinel species is subject to culling activities, for example for demographic containment, as in this case also happens for *Pica pica* in the Italian territory. This method would also allow to evaluate the degree of accumulation of THMs in the food chain, which is then the fraction that represents a danger for biological systems. This would allow us to neglect the presence of the offending elements associated with the mineral matrix, frequently encountered in minerals, but at the same time, in most cases, present in bound form and not easily reachable by biological systems. The main limit to an extensive application in spatial and temporal terms of this monitoring strategy would be of various natures. In the first place the accessibility of the samples is not immediate as it depends on capture of the subjects. Also, the analytical evaluation of the samples is complex, foreseeing the analysis with ICP-MS. Another challenge is the complexity of the method for the detailed sample preparation procedures, the high technological level of the analysis systems and the need for highly specialized technical personnel dedicated to the operation of these systems, all resulting in a high unitary cost of analysis.

Contrariwise, the evaluation of the degree of OS assessed based on OS biomarkers in sentinel subjects proved to be a bad system for indirect monitoring of the degree of exposure to THMs in field conditions. Probably the level of HMS exposure that we observed is not sufficient to determine a perceptible oxidative action or in any case discriminable by interference on OS levels associated with environmental (ex. food sources) and/or intrinsic factors (ex. immune response). The latter

factors can more clearly influence the oxidative balance of the organism, covering minor variations potentially associated with THMs chronic exposure, with micro dosages for a long time.

Based on what emerged, in particular from the second and third study, the employment of OS biomarkers evaluation in bird species seems promising as a tool for the detection and monitoring of health problems. It would also be useful to evaluate animal welfare, also in consideration of the fast execution times and low cost of analysis. In particular, the possible developments in terms of immune monitoring are interesting, with clinical applications both of a diagnostic type (highlighting of current pictures) and of a prognostic type (prediction of a given type of clinical course).

The second study showed that the levels of MDA and OSI are associated with stressful situations in hens (higher density of subjects and indoor environments with higher levels of dustiness), while in *Pica pica* was found a positive correlation between WBC leukocyte level with d-ROMs and OSI. From the third study emerged that following immune activation, leukocyte, and lymphocyte replication is not associated with antibody production, indeed it can even be negatively correlated. Speaking of immune response and OS, the study of d-ROMs has proven to be useful for predicting the course of the immune response, where, following the antigenic stimulus, subjects with higher initial levels of d-ROMs tend to develop greater cellular mobilization, with greater increases of WBC and Lym but less antibody synthesis.

It was also observed that the antioxidant component is strongly correlated to the trend of the antibody response, specifically as subjects with high levels of FRAP following the antigenic stimulus tend to develop major entity of antibody responses, and that subjects with higher antibody responses tend to develop and maintain high levels of SOD.

In conclusion, the study of oxidative stress in relation to the immune response could have interesting applications from the clinical diagnostic point of view. Also, it would be desirable to develop standardized methods to identify reference parameters.

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