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**ECOTOXICOLOGICAL STATUS OF TWO BIRD SPECIES (*Falco tinnunculus* and *Parus major*) AND THE INFLUENCE OF CHANGING ENVIRONMENTS: A MULTI-BIOMARKER AND COMPLEMENTARY APPROACH**

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**ABSTRACT**

Birds play a pivotal role in maintaining the delicate balance of ecosystems through various ecological functions. However, the growth of cities, agriculture intensification, and industrial expansion profoundly impact wild birds by modifying natural environments and releasing a wide range of contaminants that exhibit similar or synergistic, antagonist, or additive ways of action. Simultaneous exposure to multiple stressors and contaminants mixture can make their impact even more severe, inducing physiological, behavioural and toxicological responses by organisms. To monitor such complex conditions would require the application of a multi-tier approach integrating information at different levels of biological organization with data on chemical contamination and on the environment in which the different populations live.

The aim of this thesis was to: a) to conduct a broad spectrum assessment of the toxicological status of two bird species (*Falco tinnunculus* and *Parus major*) sampled in the wild using a multi-biomarker and complementary approach based on contaminant analysis, biomarkers responses and behavioural and breeding performances; b) to investigate the influence of environmental changes and multi-stressors on the physiological state/adaptation ability of the two bird species.

The thesis permitted to develop and apply genotoxicity and immunotoxicity biomarkers aiming to extend the biomarkers battery that can be used *in vivo* and *in vitro* studies on Common Kestrel, and in other bird species, enabling the measurement of fundamental endpoints for evaluating the health status of the avian populations.

*In vitro* laboratory studies on Common Kestrel blood aiming to evaluate cytotoxicity and genotoxicity of two commercial pesticides, an herbicide (Round up) and a fungicide (Amistar®Xtra), using the comet assay were performed. The comet assay, used to evaluate potential DNA damage, showed substantial differences in the genotoxic potential of the two compounds, round up (glyphosate) was found to be more toxic in comparison with fungicide. Glyphosate resulted to be strongly toxic to cells even at concentrations well below usage doses and for very short time of exposure. The developed *in vitro* approach showed interesting toxicological effects of two pesticides widely used and less investigated and the effectiveness in testing the toxicity of commercial pesticide formulations instead of the active principles.

A two-year monitoring study on Common Kestrel aiming to evaluate the ecotoxicological status and the physiological responses of populations from areas with different anthropic pressures by applying a blood-based multi-biomarker approach combined with contaminant analysis was conducted. A wide set of biomarkers was applied on blood of nestlings sampled in urban, rural and natural areas

of Lazio Region. Some of the biomarkers used, such as respiratory burst and comet assay, were developed for the first time in this species in the laboratory studies and successfully applied in this investigation. All the results suggest that the urban environment is not necessarily more risky for birds than other types of habitats. Our multi-marker approach suggests that kestrels probably face with environmental stressors that differ in typology or intensity across habitats, requiring adequate physiological responses. Indeed, important differences of contaminants concentrations among the studied environments were also found.

A three-year monitoring study on Great tit aiming to evaluate the ecotoxicological status of populations from areas with different anthropic impacts using a multi-biomarker and complementary approach was conducted. The study was carried out in wood, urban and agricultural areas of the Veneto Region. The integrated monitoring approach allowed to get a complete picture of the toxicological status of great tit populations by integrating biomarkers responses with contaminant analysis data and information on behavioral traits and reproduction success parameters. We found that the great tit populations of urban and agricultural (agri 1) areas were those with the most significant impacts resulting from environmental pressures. In addition, thanks to this holistic approach, it was also possible to identify sub-lethal effects in individuals from the other areas, showing the importance of evaluating different endpoints during a monitoring study. Although biomarker alterations emerged, no evident effects at the population level were found. However, it must be taken into account that biomarker alterations represent early warning signals of possible long-term effects for individuals and populations, therefore a longer-term monitoring is needed.

The theorized multi-biomarkers and complementary approach on wild Common Kestrel and Great Tit populations inhabiting areas characterized by different anthropic pressures was successfully applied. The developed approach enables to obtain interesting information on the impact of the different environments and thus the various stressors on the studied bird populations. Results showed the effectiveness of the multi-biomarker and complementary method in identifying different biological and toxicological responses/adaptation due to environmental stressors that differ in typology or intensity across habitats. This information is particularly relevant to guide exploitation of lands and urban planning in a more compatible way with the health of wildlife. Moreover, the complementary approach applied in this study could be applied to monitor other bird species considered as targets. Furthermore, this approach can be proposed also at managemental and regulatory level for the conservation and protection of wild and endangered bird species.

**STRUCTURE OF THE THESIS**

This thesis is structured in five chapters.

The first chapter is a general introduction presenting different avian ecotoxicology topics. In detail, this chapter describes the importance of birds as sentinel species, the state of birds worldwide, the threats to which these animals are subjected, the importance of biomarkers in the field of avian ecotoxicology, and finally, the biological meanings of the applied biomarkers and the behavioral traits and reproductive success parameters investigated in the dissertation.

The second chapter describes the main aims of this work of thesis.

The third chapter concerns the ecotoxicological studies on *Falco tinninculus*, Common Kestrel. This chapter includes an introduction represented by a published review paper that investigates the gaps of knowledge on the toxicology of these raptors and the methods used or suggested to evaluate the ecotoxicological effects on Common Krestel individuals (Giovanetti L., Casini S., Campani T., Caliani I., 2023. State of the art, gaps, and future perspectives on Common Kestrel ecotoxicology. Environmental Toxicology and Pharmacology. DOI: 10.1016/j.etap.2023.104237). The aim of this chapter is included. Other sub-topics are: development and application of genotoxicity and immunotoxicity biomarkers on blood of Common Krestel; *in vitro* laboratory studies in which blood Kestrel samples were exposed to a fungicide and an herbicide, and genotoxicity effects were evaluated using the comet assay developed were also performed. This chapter also includes a published paper of a monitoring study conducted on Common Kestrels populations from different areas of the Lazio Region, characterized by different anthropic pressures. The monitoring activities were conducted during 2020 and 2021, and the status of the kestrel populations was assessed using a blood-based multi-biomarker approach, to which chemical analysis of kestrel feathers and eggs were integrated. The pdf of the published paper is reported (Giovanetti L., Caliani I., Damiani G., Dell'Omo G., Costantini D., Casini S., 2024. A blood-based multi-biomarker approach reveals different physiological responses of common kestrels to contrasting environments. Environmental Research. DOI: 10.1016/j.envres.2024.118674). The conclusions of the ecotoxicological studies on Common Krestel are also presented in the chapter.

The fourth chapter concern the ecotoxicological studies on specimens of *Parus major*, Great tit. This chapter describes the state of art on the ecotoxicology of Great tit and a monitoring study conducted on great tit populations from different areas of the Veneto Region, characterized by different anthropic impacts. The monitoring campaigns were carried out for three years (2021-2023) and the

ecotoxicological state of great tit populations was evaluated using a multi-biomarker and combined approach integrating chemical data, biomarkers responses, behavioural and breeding information. Conclusions of these studies are included.

The fifth chapter contains the final conclusions, aiming to summarize the main findings of the laboratory and field studies conducted on the two avian species.

# CHAPTER 1
## INTRODUCTION

# CHAPTER 1

## INTRODUCTION

### 1.1. Avian ecotoxicology

Worldwide, the environment is negatively affected by anthropogenic activities. Urbanization, industrialization, and agricultural activities release a wide range of pollutants into the environment that can cause temporal discomforts or permanent damages to biota, birds included (Dutta, 2017; Ding et al., 2023). In this context, avian ecotoxicology combines different sciences from chemistry, toxicology, environmental sciences and biology, to study the movement of environmental contaminants through ecosystems and then to evaluate their lethal and sub-lethal effects on birds (García-Fernández, 2014). Avian ecotoxicology allows to assess the contaminant toxic effects at the individual level and to link them at the population level. Ecotoxicological methods are sensitive, allow to study the contaminants "in mixture" and provide a more realistic biological information of the toxic effect on the health status of organisms and, therefore, of the entire ecosystem (Oost et al. 1996).

Birds play an important role in natural ecosystems and have a certain ecological relevance due to their ecosystem services. Indeed, they perform important functions such as predation, seed dispersion, pollination, scavenging, and as ecosystem engineers (Whelan et al., 2008). Thanks to their features, since the ’60s, birds have been used in ecotoxicological studies and are considered good bioindicators of environmental health. Birds are, among vertebrates, the group most sensitive to environmental contamination, as they generally occupy high trophic levels and are distributed worldwide (Hao et al., 2021). Their apical position in the food chain makes them susceptible to bioaccumulation and biomagnification processes (Francis, 2017; Baesse et al., 2019), and their wide distribution exposes them to anthropogenic contaminants present in the different environmental compartments and matrices (Khorami et al., 2017). The avian species are good sentinels of environmental quality because they can be studied rather easily in the field. Breeding parameters, development phases, and behavioural traits can be recorded (Smits e Fernie, 2013). Moreover, especially nestlings, are relatively easy to capture, allowing for sampling non-destructive biological material such as blood, feathers, excreta, and unhatched eggs (García-Fernández, 2014). Finally, the broad knowledge of birds’ physiology, ecology, and behaviour makes the development of experimental designs easier, as well as the comprehension of results (Jaspers et al., 2015).

### 1.2. The state of world's birds

The number of bird species in the world is approximately 11.000. However, the global data of the Red List of Birds, updated to 2022, are in many ways worrying. Globally, almost half of the bird species are declining and, 1400 species are considered threatened, specifically: 233 species are listed as critically endangered, 413 as endangered, and 754 as vulnerable (iucnredlist.org). What is important to underline is that the world's birds decline affects not only the threatened species but also the widespread and common ones. The decline rate of common species is not so high to include them among those threatened, but their monitoring remains essential to ensure their conservation and prevent possible effects on the ecosystem (BirdLife International, 2022).

At European level, the situation is not less alarming, in fact since 1980, about 600 million of birds have been lost. In EU, 86 of bird species are inserted in the IUCN red list, with 9 species classified as critically endangered, 21 species as endangered, and 56 species as vulnerable (BirdLife International, 2021). Considering the birds on the basis of their habitat, the Pan-European Common Bird Monitoring Scheme reported a reduction of all common avian species in the European Community of 18 % from 1980 to 2021, with the highest rate of decrease (60%) of common farmland birds (pecbms.info).

### 1.3. Threats to birds

The pressures responsible for the deterioration of the world's bird species are mainly linked to human activities. The principal threats impacting birds include agricultural expansion and intensification, logging, pollution, invasive species and climate change (Richard et al., 2021; datazone.birdlife.org). In addition to these pressures, other important menaces come into play, such as overexploitation (hunting and trapping), infrastructure development (expansion of residential and commercial areas, energy production and transports), wildfires, and even bycatch from fisheries (BirdLife International, 2022). These multiple pressures can cause loss of important bird habitats, disturbance, increasing competition, reproductive success reduction, and sometimes death (Shipley et al. 2020).

Agricultural, logging and infrastructure development are key drivers, especially agriculture, of habitat degradation, fragmentation and simplification and, therefore, of bird population decline (Donald et al., 2006; Emmerson et al., 2016; Xu et al., 2018; Leaver et al., 2019; Traba and Morales 2019; Hendershot et al., 2020; Morgado et al., 2020; Balotari-Chiebao et al., 2023; Douglas et al., 2023; Lloyd et al., 2023; Mills et al., 2023; Pavlov et al., 2023; Rabbetts et al., 2023).

The increase of the world's population and food demands determine a conversion of fallow land or grassland to cropland and currently 40% of the world's territories are converted to agricultural use (ww.fao.org). These factors can affect avian populations due to habitat loss, pesticides toxicity, and the reduction of food sources (Stanton et al., 2018; Mineau and Whiteside, 2013; Reif, 2013).

The use of plant protection products (PPP), such as insecticides, herbicides, fungicides, acaricides, nematicides, molluscicides, rodenticides, etc., is the main source of pollution in the agricultural sector. Already in sixties, deleterious effects of pesticides on birds are being presented to the general public by Rachel Carson in the book 'Silent Spring'.

Birds are non-target organisms that suffer direct and indirect effects from exposure to pesticides (Sala et al., 2010; Mitra et al., 2011) and common farmland birds have been the most threatened species since 1980. Pesticide effects can occur, directly or non-directly, at different level of biological organization. PPP can cause direct toxic effects on the physiology and behaviour of birds, can impair their survival and reproduction and, therefore the population dynamics (Stanton et al., 2018). Also, variations in immune function, oxidative parameters and genetic alteration represent sublethal effects at the cellular/biochemical level determined by PPP exposure. Reduction in cellular immune responsiveness was found in offspring of red-legged partridge adults (*Alectoris rufa*) exposed in a realistic scenario to thiram and imidacloprid-treated seeds (Lopez-Antia et al. 2015a; Lopez-Antia et al. 2015b). Another laboratory study conducted on quails treated with an organophosphate pesticide (chlorpyrifos) showed an increase in the concentration of malondialdehyde, glutathione, nitrite, hydrogen peroxide and activity of superoxide dismutase; thus chlorpyrifos caused oxidative stress in quails (Ćupić Miladinović et al., 2018). Breakage of DNA in germ cells were registered by Tokumoto and collaborators (2013) in mature male quails treated orally with a neonicotinoid (clothianidin). Carbamates and organophosphates inhibit acetylcholinesterase (AChE), the principal enzyme in the nervous system (reviewed by Mitra et al., 2011). Embryo's brain of Japanese quail (*Coturnix japonica*) can be damaged after exposure to glyphosate-based herbicides (Ruuskanen et al., 2020). The effects of pesticides on nervous system can also induce behavioural alteration in exposed animals (Moreau et al., 2022b). In addition, they can also act on reproductive success by altering egg laying, clutch size, egg size and eggshell thickness, egg fertility and brood size (Lopez-Antia et al. 2015 a; 2015 b; 2018; 2021; Kumar et al. 2023).

Indirect effects of PPP occur as a fundamental reduction of habitat and food supply, especially during the breeding season. Brickle and collaborators (2000) showed how the increased use of pesticides in the UK reduced the availability of invertebrates, a food source for the bird species, *Miliaria*

*calandra*. The same study also found a modification of the chicks' feeding, nourished mainly with cereals instead of insects; thus with an inadequate food for a proper development. Similarly, another study showed that the use of herbicides caused a reduction in the availability of weed seeds, a food included in the diet of different bird species (Gibbons et al., 2006).

Also in urban environments birds are subject to various pressures that include habitat loss and fragmentation, pollution, disturbance and collisions with buildings (Herrera-Dueñas et al., 2014; Dri et al., 2021; Elmore et al., 2021; Saulnier et al., 2023). In addition, they are negatively impacted by light and noise pollution (Cabrera-Cruz et al., 2018; Dutta, 2017). For flying, birds accumulate large volumes of air and absorb gases or particles present in the air (Brown et al., 1997; Baesse et al., 2019), therefore, they are highly vulnerable to chemical pollutants present in urban areas (Sanderfoot and Holloway, 2017). Vehicle traffic, home heating and other human activity are sources of severe air pollutants (like nitrogen and sulfur oxides, ammonia, particulate matter, heavy metals, polycyclic aromatic hydrocarbons).

Urban contaminants may cause sublethal effects at different levels, from molecular and biochemical to individual and population and not last, the death of the animals (Barton et al., 2023). For example, air pollutants can reduce telomere length in wild *Parus major*, lead to thyroid function alteration in American kestrel (experimentally exposed to NO2, SO2, benzene and toluene) and increase the antioxidant capacity of urban passerine species exposed to nitrogen oxides, which are potent pro-oxidants (Grunst et al., 2019; Fernie et al., 2016; Salmón et al., 2018). Contamination from heavy metals can have immunotoxic effects. Specifically, heavy metals can alter the white blood cells composition unbalancing the ratio between heterophil and lymphocyte (H/L), as shown by (Bauerová et al., 2017). Genotoxic effects were recorded in passerines inhabiting peri-urban areas, with a micronuclei average number higher than birds from less polluted areas (Baesse et al., 2015). Similarly, specimens of *Phalacrocorax auritus* from the Canadian Hamilton Harbor have shown increased DNA microsatellite mutations due to high levels of polycyclic aromatic hydrocarbons (PAHs) (King et al., 2014). Moreover, the ozone may induce lung structural and biochemical variation in exposed Japanese quails (Rombout et al., 1991). The effects of these substances can occur at the behavioural level too, with a reduction in the singing repertoire of male passerine birds from areas polluted with heavy metals (Gorissen et al., 2005), as well as cause the impairment of reproductive success in birds exposed to contaminants of urban and industrial emissions (Sanderfoot and Holloway, 2017).

Focusing on industrial emissions, the damage caused to birds by some contaminants such as heavy metals, polychlorinated biphenyls (PCBs) and brominated flame retardants (BFRs) are particularly evident (Fernie et al., 2001; Ottinger et al., 2009; Harris & Elliott, 2011).

More attention should be paid to substances that have shown adverse effects on humans and wildlife in relatively recent years, namely per- and polyfluoroalkyl substances (PFAS). Some PFASs, such as perfluoro octane sulfonic acid (PFOS) and perfluorooctanoic acid (PFOA), as well as several BFRs compounds, have been already added to the listed Stockholm Convention on persistent organic pollutants (POPs) between 2009 and 2019 (chm.pops.int; Mortimer e Reichelt-Brushett 2023).

A recent study on caged canaries (*Serinus canaria*) treated with environmentally relevant concentrations of PFOS and PFOA showed transcriptomic effects on the adipose tissues. Nevertheless, no effects on physiological and immunological parameters (e.g., body weight, fat index and cell-mediated immunity) were recorded (Lopez-Antia et al., 2023).

Furthermore, high levels of PFAS can also affect birds' reproduction, consequently altering the dynamic population of species living nearby fluorochemical production plants. Indeed, Groffen et al. (2019) found a correlation between high concentrations of PFDA and a reduced hatching success, advanced egg laying, and a reduction of breeding success in *Parus major* eggs.

As a general consequence of human activities, climate change negatively impacts avian populations and significantly affects the biodiversity (Dunn and Møller, 2019). Moreover, it is interconnected with the other menaces, making their impact even more severe. As a result of climate change, birds react by modifying some crucial phases of their life. Thus, this kind of pressure modifies their distribution, abundance, and behaviour (datazone.birdlife.org). Birds can respond by shifting to different latitudes or altitudes than their original habitat. An example is the case of some high-altitude bird species in the Peruvian mountains, which have suffered a sharp decline in their suitable habitats (Freeman et al., 2018). In addition, birds can change the timing of migration and breeding. Different studies showed that migratory species frequently exhibit earlier arrivals at their breeding areas and initiate egg-laying earlier in response to elevated temperatures during the season (Usui et al., 2017; Koleček, et al., 2020; McLean et al., 2022). The anticipation of laying dates can affect the breeding success (Shipley et al., 2020) and this condition is probably related to mismatches between predators and prey availability (Møller & Berthold, 2010; Renner and Zohner, 2018; Mayor et al., 2017). However, other works found advanced laying dates but no alterations in the offspring production (Dyrcz and Czyż, 2018; Clark et al., 2014). It should be noted that some species may have advantages in

anticipating the breeding season; indeed, some research has revealed increased nestlings' production in warmer seasons (Wegge and Rolstad, 2017; Hoover and Schelsky, 2020).

### 1.4. Biomarkers as diagnostic tools

To understand and address issues arising from environmental contaminants or their mixtures, it is necessary to investigate their mechanisms of action within an organism. In the ecotoxicology field, extremely important tools, known as biomarkers, have been developed to assess the exposure and effects of environmental contaminants on biota. Biomarkers indicate a rapid response to toxicant exposure and provide information on the cumulative effects of a mixture of chemical compounds. Furthermore, they allow the integration of different episodes of exposure in time and space and are an early warning signal of possible long-term effects (Fossi & Leonzio, 1994).

When an organism is exposed to contaminants, it is crucial to consider its complexity of responses. Individuals can have alterations at different biological levels, ranging from biochemical and cellular modifications at the individual level, to behavioural changes within a population. Thus, different biomarkers can be evaluated, including DNA alterations, protein responses, metabolic products and alteration, changes in the immune system, histopathological, morphological, physiological and behavioural changes, providing effect information at different levels of biological organization. Therefore, biomarkers represent a valuable diagnostic tool that allows to assess the ecotoxicological status of an organism and to evaluate the environmental health status in which it lives.

The increase of avian threatened species have necessitated the development of non-destructive biomarkers that can be detected without causing damage or sacrifice to the animal. These biomarkers not only solve ethical problems, but also are useful in studying populations with a limited number of individuals and to detect sublethal exposure effects (Chaousis et al., 2018). Furthermore, non-destructive biomarkers are advantageous for monitoring larger numbers of specimens and for repeating sampling several times over time, even on the same specimen or population. When working with non-destructive biomarkers, it is essential to use those biological materials that are suitable for analysis, such as blood, skin biopsies, antlers, milk, feathers, excreta, faeces and abandoned eggs. The advantage of this material is that it can be sampled without harming the animals and allows the measurement of a wide range of biomarkers and residue levels (Fossi et al., 1999; Casini et al., 2003; García-Fernández et al., 2013; Espín et al. 2016; Casini et al., 2018; Movalli et al. 2018; Morganti et al., 2021; Bianchini et al., 2022).

In this study research, a multi-biomarker approach and non-destructive biomarkers were applied using appropriate biological matrices such as blood, feathers, excreta and eggs. The selected non-destructive biomarkers were able to provide different toxicological responses. Specifically, oxidative stress, porphyrins levels, immunotoxicity, genotoxicity and neurotoxicity biomarkers were evaluated. All the biomarkers applied in this study are described in detail below.

***Biomarkers of oxidative stress***

Metabolic and physiological processes occurring within an organism, such as products of mitochondrial metabolism and immune defence (Azad et al., 2010; Koivula and Eeva, 2010), produce reactive oxygen species (ROS), both radical and non-radical (·O2-, ·OH, HO2·, H2O2, RO2·, RO·).

The ROS, being highly reactive (Sánchez-Virosta et al., 2019), can bind to membrane lipids and triggering a cascade of oxidative reactions, they impair the membrane integrity in a process known as lipid peroxidation (LPO). To inhibit the formation of these radicals and prevent potential damage, these molecules are removed by enzymatic complexes such as glutathione-S-transferase (GST), catalase (CATs), and superoxide dismutase (SODs), or non-enzymatic molecules like glutathione (GSH), metallothionein, and uric acid, as well as exogenous antioxidants such as vitamin C and E, carotenoids, and phenols (Cohen et al., 2007; Costantini, 2008). These enzymes and molecules transform ROS into less reactive compounds. For instance, GSH directly binds to ROS, converting them into less harmful compounds. SODs, CATs, and GST convert other ROS into non-reactive species, making them no longer harmful to the organism (Kanwal et al., 2020). If the antioxidant complex fails to perform its function or if an organism produces or encounters excessive ROS, the animal experiences oxidative stress (Erel et al., 2004). Oxidative stress comes from ROS production and antioxidants imbalance, in favour of the first can cause damage to macromolecules and disruption of membrane homeostatic systems. For example, the ROS can bind to DNA causing lesions such as apurinic sites, single-strand breaks or oxidation of the sugar part (D'Costa et al., 2019; de Siqueira et al., 2020; Glei et al., 2016).

Certain heavy metals (such as lead, cadmium, chromium, and copper) can induce the production of ROS species if present in large quantities within organisms (Yao et al., 2023). Even pesticides can increase LPO by inducing ROS production, which, in turn, compromise membrane functionality and integrity (Maiti et al., 1996).

Birds, generally, have the capabilities to detoxify harmful compounds and minimize oxidative stress (Costantini, 2008), primarily because they have a low ROS production at the mitochondrial level (Koivula and Eeva, 2010; Jimenez, 2018; Gomez et al., 2023).

In the present study, we assessed a potential condition of oxidative stress in bird individuals by applying the Total Antioxidant Status (TAS) assay and measuring the total glutathione (tGSH) and its reduced glutathione (GSH) and oxidase glutathione (GSSH) forms.

TAS assay or TEAC (Trolox-equivalent Antioxidant Capacity) or TAC (Total Antioxidant Capacity) allows to measure plasmatic antioxidants capable of inhibiting a redox reaction induced by free radicals (Choen et al., 2007; Bourgeon et al., 2012). This assay has the advantage of being a functional measure because allows to evaluate the overall ability of an organism to counteract oxidative stress and prevent oxidative damage to cells and tissues. However, it should be underlined that different antioxidants (such as uric acid) exhibit different kinetics complicating the interpretation of the results, especially in birds where plasma uric acid content is high and varies dramatically between species (Montgomery et al., 2012; Cohen et al., 2009; Cohen et al., 2007). An upregulation of the antioxidant defence can be adopted by birds in response to contaminant exposure; this allows birds to protect themselves against possible oxidative damages. An increase of TAC was showed by Fenstad and collaborators (2016) in common eiders (*Somateria mollissima*) environmentally exposed to POPs in a polluted ares of Baltic. Another study reported an increase of the total antioxidant capacity on great tit (*Parus major*) individuals living in the vicinity of a smelter plant in Belgium (Greens et al., 2009).

The glutathione (GSH) is a crucial non-enzymatic antioxidant and detoxifier of pro-oxidants (Isaksson, 2013). In a normal condition, the predominant form of GSH is the reduced form (Yao et al., 2023), but when GSH act as a scavenger, it is oxidized to GSSG. A lower GSH:GSSG ratio indicate that an organism is affected by oxidative stress (Isaksson et al. 2005; Isaksson, 2010). The balance between pro- and antioxidant of wild animals can be altered by several pressures mainly related to human activities, such as: urbanization, light and noise pollution, contamination, climate change, food quality and availability, but also immunological responses and life history strategies of an animal (Beaulieu and Costantini, 2014).

***Porphyrins levels***

Some contaminants can act on the normal synthesis of endogenous compounds. A representative example is the alteration of heme biosynthesis which can be impaired after exposure of an organism to several contaminants, including heavy metals, dioxins and PAHs causing porphyrins production and accumulation.

Porphyrins are pigments, containing a tetrapyrrole porphin nucleus, widespread in nature. They are present in some animal tissues, for example in shells and feathers in birds, as pigmented depositions where they perform a function for ornamentation and camouflage. However, their fundamental physiological role is linked to the synthesis process of heme (hemoglobin prosthetic group), of which they are intermediate metabolites (e.g. protoporphyrin) or oxidative byproducts of the intermediate porphyrinogens metabolites (coproporphyrins and uroporphyrins) (Casini et al., 2003). Heme synthesis occurs through a series of reactions catalysed by different enzymes with a series of intermediate products up to protoporphyrin IX that when added a Fe atom is transformed in heme. Porphyrins are produced and accumulated in traces in the erythropoietic tissues, specifically in erythroid cells. In the liver, heme is required for the synthesis of different hemoproteins, particularly cytochrome P450 (Marks, 1985). Other biological sites of accumulation are kidney, fur for mammals, urine and faeces. Alterations in the heme biosynthesis pathway provoke an increase in porphyrin levels, and this can be used as a non-destructive biomarker, as index of exposure to environmental contaminants (Marks, 1985). In birds, porphyrin levels can be evaluated in feathers, eggshells and excreta. Pollutants such as PAHs, PCBs, hexachlorobenzene (HCB), organophosphorus compounds (OCs), dioxins, furans and heavy metals, act very selectively on different enzymes of the heme biosynthesis modifying the pathway without though causing any major macroscopic effect, therefore porphyrins can be considered as a biomarker of exposure rather than of effect. More precisely, they represent a sensitive biomarker of exposure, considering that they can be detected in different biological materials even at very low concentrations thanks to their characteristic absorption spectrum (Casini et al., 2003).

In this study, we analysed the following types of porphyrins in excreta:

- Coproporphyrins: intermediates of heme synthesis that are normally present in small amounts in blood, urine and faeces, but tend to accumulate in different biological materials after exposure to different contaminants such as HCB, PCBs, Hg (Casini et al., 2003) and Pb (Fowler & Mahaffeyt, 1978).

- Uroporphyrins: there are four type-isomers of uroporphyrin, they constitute the pigments present in the urine, and can originate from the inhibition of the uroporphyrinogen decarboxylase. The amount of uroporphyrins excreted through urine was found to increase significantly after exposure to PCBs, dioxins, furans (Casini et al., 2003) and As (Fowler & Mahaffeyt, 1978).

- Protoporphyrins: intermediate metabolites of heme biosynthesis; the exposure to PCB compounds was found to cause accumulation of protoporphyrins in birds liver (Fossi et al., 1996a). Whereas Pb intoxications cause high levels of protoporphyrins in erythrocytes. Specifically, Pb interferes with the iron transfer mechanisms causing therefore a reduction of availability of Fe2+; in this condition Zn can substitutes iron as a substrate and forms zinc-protoporphyrin instead of heme (Casini et al., 2003). Furthermore, protoporphyrins production, as well as other porphyrins, can result from a general mechanism of oxidative stress, which can oxidize precursors of porphyrins.

Casini and collaborators (2001) showed higher porphyrins levels in excreta of brown pelicans (*Pelecanus occidentalis thagus*), neotropic cormorants (*Phalacrocorax olivaceus*), and kelp gulls (*Larus dominicanus*) individuals from industrial and agricultural areas than those from a non polluted site. Another field study conducted on mallard (*Anas platyrhynchos*) and coot (*Fulica atra*) reported a positive correlation between Pb concentration and coproporphyrins and protoporphyrins levels in excreta (Martinez-Haro et al., 2011).

***Immunotoxicity biomarkers***

The immune system of birds, similarly to that of all other vertebrates, is divided in innate and adaptive immunity. The innate immune system produces nonspecific responses that represent as an initial defence to eliminate microbes or prevent infections. Components of the innate immune system include complement system, acute-phase proteins, and phagocytes (granulocytes, monocytes, and macrophages). Additionally, in the innate immune response are involved inflammation mediators such as basophils, mast cells, eosinophils, and natural killer (NK) (Demas et al., 2011; Vallverdù-Coll et al., 2019). The adaptive immune system produces acquired immune responses specific to antigens. It can act through cell-mediated immunity, which eliminates virus-infected cells and defends against fungi, protozoa, intracellular bacteria, and tumours. Alternatively, through humoral responses, it can act against bacteria, bacterial toxins, and viruses before they enter into cells by producing antibodies (O'Neal et al., 2013). Cell-mediated responses involve

lymphocytes T (a leukocytes' class), which attack foreign cells by binding to their antigens and inducing cell death. On the other hand, humoral response perform the production of specific antibodies, called "immunoglobulins (Ig)", by lymphocytes B, directed against antigens of particular pathogens (Salvante et al., 2006).

Environmental pollution can impair the bird's immune system (Li et al., 2021; Grasman, 2002). As the immune system is fundamental for defencing against pathogens and other threats, the suppression of it, induced by contaminants, can lead to decreased host resistance, increased susceptibility to disease and therefore reduce fitness and the size of the populations (Vermeulen et al., 2015; Vallverdú-Coll et al., 2019). Thus, immunocompetence can provide a useful tool for evaluating the impact of contaminants on organisms.

In the present study we evaluated potential alteration in immunocompetence by assessing respiratory burst process, complement system activity, bactericidal capacity and leucocyte profile of bird individuals.

The respiratory burst (or oxidative burst) process plays an important role in the innate immune response and involves the rapid release of reactive oxygen species by phagocytes (Goody et al., 2013). The family of phagocytes includes granulocytes, monocytes, and dendritic cells, all originating from bone marrow stem cells. Monocytes and granulocytes carry out their phagocytic function by engulfing organisms and foreign agents, subsequently degrading them. The efficiency of these processes can be enhanced by cytokines (Mast et al., 1998). Dendritic cells, on the other hand, play a crucial role in recognizing and engulfing antigens to present them to B and T lymphocytes, serving as the most important cells within the Antigen-Presenting Cell (APC) ensemble (Zmrhal et al., 2020). Specifically, the respiratory burst process begins with the activation of the NADPH oxidase, which produces highly reactive oxygen radicals such as superoxide, hydrogen peroxide, and hydroxyl radicals. These reactive oxygen species, interacting with the pathogen, damage and make it susceptible to subsequent phagocytosis and elimination (Pasmans et al., 2001). The ROS produced during respiratory burst, are not pathogen-specific and can also damage host tissues. It can lead to immunopathology damaging the organisms macromolecules (Meitern, 2016). An alteration of the oxidative burst was found in different avian species in response to contaminants exposure (Holloway et al., 2003; Garg et al., 2004; Hansen et al., 2020).

The complement system, include nine proteins (C1 to C9) present in the plasma, which are activated to induce target cells lysis and facilitate their phagocytosis through opsonisation (Moleòn et al.,

2020; Costabile et al., 2010). Moreover, it plays a crucial role in the elimination of microorganisms and the inflammatory response by modulating the vascular permeability of immune cells, facilitating their entry into infection sites (Demas et al., 2011). The entry of a pathogen or foreign molecule into organism induce the complement system activation and can occur through three pathways: a classical antibody-dependent pathway (involving C1qrs, C2, and C4 proteins), an alternative antibody-independent pathway (involving C3, factor B, and properdin), and a lectin pathway (Merchant and Britton, 2006). These pathways lead to form the Membrane Attack Complex (MAC). The MAC binds to the surface of microorganisms or abnormal cells, creating pores on their surface, ultimately leading to their lysis (Frye et al., 2020; Yu et al., 2021). A higher lysis indicates a robust immune response (Vennum et al., 2019).

Natural antibodies (NAbs), along with the complement system, constitute components of the innate immune system, playing a fundamental role in the initial defense against pathogens. NAbs are predominantly isotypes of immunoglobulins IgM, IgA, and IgG, engaging in multiple functions. Specifically, they can remove foreign molecules, dead pathogens, or catabolic products. They provide initial protection against infection, facilitate the antigens attachment to B lymphocytes, and contribute to enhancing specific immunity (Lammers et al., 2004; Parmentier et al., 2004). Furthermore, NAbs are involved in opsonizing pathogens and initiating the enzymatic cascade of the complement system. These antibodies are capable of agglutinating and lysing foreign cells. Agglutination is an interaction between antigen and antibody that produces antigen aggregates that precipitate making foreign cells harmless (Matson et al., 2005).

The leukocytes, commonly known as white blood cells (WBCs), are formed in various organs and during different birds life stages. During embryonic development, hematopoiesis occurs in the yolk sac until it stabilizes in the bone marrow. The spleen also contributes to their formation until the eighteenth day of life. In birds, T lymphocytes mature within the thymus, while B lymphocytes differentiate in another lymphatic organ above the cloaca, known as the bursa of Fabricius (Claver et al., 2009).

There are different classes of leukocytes, distinguished by structure and function:

- Heterophils are recognizable by their colourless cytoplasm and rod-shaped eosinophilic granules. Functionally, they resemble neutrophils in mammals, migrating to inflammation sites and eliminating pathogens through phagocytosis.

- Eosinophils display a light blue cytoplasm with round granules inside. Although their function is not entirely clear, they are recruited following the introduction of foreign antigens.
- Basophils are identified by a round nucleus and violet-reddish granules, smaller than those of eosinophils. They play a crucial role in early inflammation.
- Lymphocytes are among the most abundant leukocytes in some bird species, featuring a regular shape with a spherical nucleus and basophilic cytoplasm. Their role is regulating cell-mediated responses and antibody production.
- Monocytes have a rounded shape with a kidney-shaped nucleus, and their cytoplasm is blue or bluish-gray with often a pink granular area. They function as macrophages (Claver et al., 2009; Duan et al., 2019).

The total number of leukocytes increases in the presence of pathogens within the organism. High concentrations of toxic elements, such as heavy metals, when taken in sub-lethal doses, can cause anemia and an overall increase in the white blood cell count, often associated with a high number of heterophils (H) and a decrease in lymphocytes (L), resulting in an elevated H/L ratio. Higher H/L ratio indicates potential acute intoxication, long-term stress, or an inflammatory event (Bauerovà et al., 2020). In a study conducted on common kestrels, leukocyte values were analysed, demonstrating that in situations of severe stress, the organism increases corticosterone production, leading to an elevated H/L ratio (Müller et al., 2011).

***Genotoxicity biomarkers***

The genome can be damaged by several factors, included anthropic contaminants. Similarly, environmental changes with rising temperatures can compromise DNA stability (Santos et al., 2017). Persistent organic compounds (such as PAHs, OCs, PCBs, dioxins and furans) are highly toxic substances that represent an high risk for animals due to their difficult degradation (Edulijee et al., 2001). These contaminants are able to bind directly to DNA molecules, changing their structure and integrity. Sometimes, during metabolic processes, some of these contaminants can be converted into intermediate compounds that exhibit a higher genotoxic capacity compared to the original compound (Shugart et al., 2000). For instance, benzo(a)pyrene, DDT and PCBs, form adducts by covalent binding with DNA molecules after being metabolized into more reactive intermediates (Ching et al., 2001; Østby et al., 2005). Many other compounds cause the formation of free radicals or abasic sites resulting in the breakdown of phosphodiester bonds within DNA molecules (Shugart et al., 2000). Also, heavy metals can cause direct or indirect damage to the nucleated erythrocytes

of birds, fish and amphibians, causing nuclear abnormalities by modifying the cytoskeleton and the ion membrane permeability (De Mas et al., 2015; Farag et al., 2018). Among heavy metals, mercury (Hg), can alter the mitotic spindle leading to chromosomal aberrations such as aneuploidy (change in the number of chromosomes) and polyploidy (higher than normal number of chromosomes) and nuclear abnormalities, such as micronuclei (MN) (Guilherme et al., 2008). Cells can implement DNA repair mechanisms and, when these are not sufficient, the damage can persist and cause mutagenesis (modification of gene information), teratogenesis (malformations in development), clastogenesis (breakdown of the chromosome) and finally, in the most severe form, carcinogenesis (formation of tumors or neoplasms) (Siu et al., 2004).

In this study we evaluated potentially genotoxic effects on bird individuals applying two different tests: the comet assay and the Erythrocyte Nuclear Abnormalities (ENAs) assay.

The comet assay (o single-cell gel electrophoresis) is a sensitive method that allows to evaluate DNA damage at the level of a single cell. This test permits to investigate the presence of single or double-strand breaks, alkali-labile sites, altered bases, incomplete repairs, and cross-links between the two DNA strands, and also a condition of programmed cell death (apoptosis). Alkali-labile sites are sugar residues without bases that act as intermediate products in the repair of damage, whereas cross-linking is a process occurring when exogenous molecules covalently bind with two DNA nucleotides, either within a single strand or between opposing strands (Collins et al., 2009; Jha et al., 2008). When these damages are not repaired, they can lead to more severe consequences that affect different levels of biological organization: from cells, organs, the entire animal, up to the populations of a given species. The latter condition may occur when mutations reduced growth and embryos and adults' survival (Caliani et al., 2014).

The ENAs assay allows the evaluation of chromosomal abnormalities affecting the nucleus of red blood cells (Farag and Alagawany, 2018). These abnormalities occur during the cell's mitotic division due to chromosome breakage or a chromosome's failure to properly reach the mitotic spindle (Herek et al., 2021) or can also be caused by increasing permeability of the nuclear membrane due to interaction with contaminants (Viana et al., 2018). Some authors report that the different forms of nuclear anomalies may be a consequence of the damaged region of DNA that while being moved outside the nucleus to be eliminated by exocytosis, generates the presence of imperfections on the membrane (Herek et al., 2021; Fenech et al., 2011).

***Neurotoxicity biomarkers***

Neurotoxicity effects can be evaluated by measuring the esterase enzyme activities. Esterases belong to the class of hydrolases (Shabbir et al., 2022) and are classified into esterases of type "A" and "B." The first one is responsible for organophosphates detoxification while the latter are inhibited by these pesticides (Fossi et al., 1992). B-esterases include acetylcholinesterase (AChE) and butyrylcholinesterase (BChE), defined as cholinesterases –ChEs, and the family of carboxylesterases (CaEs) (Solé et al., 2022). The inhibition of brain (AChE) and blood (BChE and CaE) esterases is an exposure and/or effect biomarker for the presence of organophosphate (OPs) and carbamate (CBs) pesticides. Indeed, due to their function in hydrolyzing esters into alcohol and an acid, esterases can bind to toxic compounds with an ester group in their chemical structure, such as OPs and CBs that cause inhibition of B-esterases (Santos et al., 2012).

In this study the activity of BChE and CaE was measured in bird plasma. They are not related with direct neurological effects of anti-cholinesterases pesticides (unlike AChE) but they can provide an early response to the exposure of OPs and CBs insecticides (Sanchez-Hernandez, 2007; Fossi et al., 1992) and their inhibition can be indirectly related to AChE inhibition (Fossi et al., 1992; Fossi and Leonzio, 1994).

BChE is synthesised in the liver, secreted into the plasma blood and distributed in different body compartments; in birds it is present mostly at the plasma level (Tsarpali et al. 2020). The evaluation of BChE activity in bird plasma is a sensitive biomarker of indirect effect of neurotoxic compounds. Indeed, a correlation between the inhibition of BChE in plasma and AChE in brain was found in the experimental study conducted by Fossi and collaborators (1992) on Japanese quails. BChE is involved in preventing inhibition of AChE, thus it is considered a system of protection of this enzyme (Omedes et al., 2023). This enzyme is inhibited by OP and CB pesticides but, in other animals, it was found to be inhibited also by PAH compounds, probably through a less specific mechanism (Casini et al., 2006).

CaEs are widespread enzymes primarily responsible for detoxifying a range of endogenous and exogenous ester compounds, OPs and CMs included. These enzymes have a dual role: safeguarding against the inhibition of AChE by offering alternative binding sites for insecticides and participating to phase I metabolism by converting substances into more soluble forms for easier excretion (Casey Laizure et al., 2013). It should be underlined that some studies found a significant impact of body

size, diet and sex in plasma ChEs and CaE activities in birds (Fossi et al., 1996b; Roy et al., 2005); therefore, these aspects should be considered when evaluating esterase activity in birds.

As mentioned above, it has been shown that several stressors (e.g., habitat changes, chemical, light, and noise pollution) resulting from human activities can also affect the behavioral traits and reproductive performance of birds. We investigated these aspects to evaluate effects even at higher levels of biological organization, namely at the individual and population levels.

**1.5. Birds behaviour**

To study inter-individual behavioural variations, it is possible to assess personality traits in animals such as dominance tendency, exploratory behaviour, or emotional reactivity. Emotional reactivity can be quantified through various tests, including measurements of respiratory frequency, pecking frequency, alarm calls, or escape attempts. Additionally, the Tonic Immobility (TI) test can be employed (Calandreau et al., 2011).

In the present study we evaluated the behavioural traits of bird individuals measuring their breath rate, assessing their agitation state and their anti-predatory behaviour.

The breath rate is considered a proxy of the emotional state and agitation during the response to stress. It is associated with future exploratory behaviour in a new environment (personality trait). An higher breath rate is associated with a fearful state of the animal (Fucikova et al. 2009; Brommer & Kluen 2012; Corti et al. 2017).

The agitation state measurement is a test considered as a measure of nestlings aggressiveness and agitation (Corti et al., 2017).

Tonic Immobility (TI) refers to the spontaneous adoption of a death-feigning behaviour by an individual in the presence of a predator. The individual assumes an inert posture, entering a catatonic state that reduces the likelihood of being attacked by the predator. If the duration of the inert state increases, the probability of receiving further attacks may be reduced (Gallup, Nash, and Wagner, 1971). In birds, as demonstrated by Jones (1986) in domestic chickens, tonic immobility involves reduced vocalization, changes in heart rate and respiration, stiffness, and intermittent or total eyelid closure. This predation response can vary depending on the origin of the studied individuals, as the environment they inhabit strongly influences their behaviour (Dingemanse et al., 2012). Moreau et al. (2022a) reported that individuals from areas with intensive agriculture have a shorter average of TI duration compared to those sampled in areas with organic farming. The possibility that such behaviours differ among individuals from populations exposed to different

anthropogenic pressures makes the study relevant. Therefore, understanding the underlying causes of these variations, especially those potentially correlated with the environmental conditions in which the animal lives and develops, becomes crucial.

### 1.6. Reproductive success of avian species

Reproductive success, as defined by ornithologists, refers to the percentage of eggs or nests within a population that successfully produces offspring (Murray 2000). This is determined through numerical parameters based on the reproductive aspects of the organism, in order to compare with other populations and their respective habitats. Clutch size, the number of eggs laid by an individual, is primarily compared to the number of hatched chicks (brood size) to calculate hatching success, as demonstrated by Bailly et al. (2016). This author reported a decrease in hatching success in more urbanized environments. Fledging success, the number of chicks that leave the nest (May and Robinson, 1985), is another fundamental parameter to evaluate species' reproductive success. Diverse authors reported a decrease of fledging success in urban and agricultural areas (Riddington and Gosler, 1995; De Satgé et al., 2019; Wawrzyniak et al., 2020; Britschgi et al., 2006).

Furthermore, indicators of reproductive success include laying date and egg hatching date, as evidenced in studies on great tit populations in various European environments (Bellavita, 1991).

The birds reproductive success can be influenced by intrinsic or extrinsic factors to the animal and attributable to its habitat. Intrinsic factors include the individual's age and experience, influencing partner choice or nesting site selection, as well as health, significantly impacting fertility (Wawrzyniak et al., 2020). Extrinsic factors generally refer to habitat quality, encompassing food availability, habitat structure (tree composition), and environmental conditions. All these factors play a crucial role in determining an individual's phenology (Przybylo, Wiggins, and Merila 2001).

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**Relevant websites:**

https://www.iucnredlist.org/statistics
https://pecbms.info/trends-and-indicators/indicators/
http://datazone.birdlife.org/sowb/pressure/theme1
www.fao.org
www.chm.pops.int
www.datazone.birdlife.org

# CHAPTER 2
# AIM

# CHAPTER 2

## AIM

Birds occupy a critical ecological niche in global ecosystems, performing essential functions such as plant pollination, seed dispersal, nutrient cycling, soil enrichment, and pest regulation. The increasing urbanization, agriculture intensification, and industrial expansion significantly affect avian populations by modifying their habitats and releasing a wide range of contaminants into the environment. These stressors, especially contamination, can induce not only the death of individuals but also sub-lethal effects such as physiological and behavioural changes, and reproductive success impairment. Thus, stressors can alter the health status of birds and, over time, affect the conservation of the more sensitive avian species.

As stated above, wild bird conditions can be compromised due to their simultaneous exposure to multiple stressors that are strongly interconnected, possibly also acting in synergy, making their impact even more severe. Moreover, another crucial aspect can come into play: wild birds are often exposed to a mixture of contaminants that may exhibit ways of action similar or synergistic, antagonist, or additive interactions, exacerbating a given situation. These aspects determine very complex biological and toxicological responses by organisms, which can be investigated by applying a multi-biomarker and complementary approach that integrates information at different levels of biological organization, from the molecular and cellular and in general sub individual level, to the individual and population, including data on chemical contamination and on the environment in which the different populations live.

Thus, there is a need to create a monitoring protocol applicable on a large scale and feasible at the regulatory level.

Based on our knowledge, a multi-biomarker and combined approach has been used in birds rarely and often in an incomplete way, measuring only some biological responses or a few other parameters.

Moreover, despite the wide distribution of contaminants in the environment and the massive use of pesticides, some of their toxicological and biological consequences are unknown, and others should be studied more in depth. For example, there is a lack of information on some classes of pesticides such as herbicides and fungicides, and on several other compounds known as emerging contaminants.

Pesticide use is the predominant stressor that negatively affects birds from agricultural habitats. Nevertheless, monitoring studies are often limited to investigate lethal effects not considering the sub-lethal ones, although they can have pernicious effects even at the population level and their evaluation provides information on early response of the organism to stress condition. Moreover, to date field studies on farmland and grassland birds are still a few.

Birds from urban environment are exposed to new challenge induced by chemicals, noise and light pollution, alteration of dietary habits and disturbance by humans. Bird physiological responses, essential to cope with these urban stressors, often show mixed responses to urbanisation. Thus, there is a growing need of studies for understanding how birds adapt to urban conditions, by evaluating their physiological state.

Literature also shows a lack of *in vitro* studies on bird species. This type of study offers many advantages, such as assessing the sensitivity of different biomarkers to several compounds, animal-use reduction and use of not or low-invasive matrices. Sensitive and effective biomarker batteries can be developed through *in vitro* studies in order to be applied for monitoring studies on wild birds.

Laboratory studies can give important information on the toxicity of certain commercial compounds, depending on their use and regulation. Indeed, toxicity of commercial formulations is rarely investigated; what is being studied are lethal effects of active principles ignoring the fact that additive substances present in commercial products can also contribute to adverse effects to non target species.

The bird species investigated in this study are the Common Kestrel (*Falco tinnunculus*) and the Great Tit (*Parus major*). They are cavity nesting species that also exploit nest boxes, making catching and sampling of nestlings easier. In our regions, they are predominantly sedentary, inhabiting environments of our interest, such as agricultural, rural, and urban. Their territoriality allows for punctual information on a territory, without variables deriving from the migration. Common kestrel, being a top predator, can bioaccumulate persistent contaminants and give information about biomagnification processes. At the same time, the great tit has particular ecological relevance and is an excellent representative of the broader bird order, the passeriforms.

Nest boxes of Common Kestrel are located in areas of the Roman countryside and the city of Rome, while those of Great Tit are installed in areas of Padua city and in its countryside. Study areas are characterized by different anthropic impacts.

The main aims of this study are: a) to conduct a broad assessment of the toxicological status of two bird species (*Falco tinnunculus* and *Parus major*) using a multi-biomarker and complementary approach based on contaminant analysis, biomarkers responses and behavioural and breeding performances; b) to investigate the influence of environmental changes and multi-stressors on the physiological state/adaptation ability of the two bird species.

To achieve this goal, the specific objectives of this study are:

- To develop biomarkers of genotoxicity and immunotoxicity, selected for their ability to provide salient toxicological responses, for their applicability both for *in vivo* and *in vitro* tests, and their sensitivity to evaluate the toxicity of contaminants such as pesticides. These tests, seldom or never used before in our target species, are meant to extend the biomarkers battery selected for this study, consisting of several ecotoxicological biomarkers already well established for birds;
- To validate, through laboratory studies, the sensitivity of the developed biomarkers. To evaluate, *in vitro*, the toxicity, for not target bird species, of commercial formulates of herbicides and fungicides widely used in agriculture, also in view of better understanding field studies results; To theorize and apply a multi-tier approach combining data of contaminant analysis, biomarker responses, behavioural traits and reproductive success. Specifically, the complementary approach aims at integrating chemical analysis with other endpoints encoding for effects at different levels of biological organization: from molecular/biochemical or sub-individual levels, evaluating oxidative stress, porphyrin levels, effects on immune system, neurotoxic and genotoxic effects, to individual level, investigating nestlings' personality and agitation state, up to population level, evaluating reproductive success parameters such as hatching and fledging success.
- To apply the theorized complementary approach on wild Common Kestrel and Great Tit populations inhabiting areas characterized by different anthropic pressures. Thus, to evaluate the impact that the multiple stressors characterising the different study areas has on the studied bird populations but also to assess the possible different biological and toxicological responses/ adaptation of these populations to the different environments.
- Lay the groundwork for a broader application of the proposed complementary approach on other birds' species considered as targets and to also use it at the managerial and regulatory level.

# CHAPTER 3

# ECOTOXICOLOGICAL INVESTIGATIONS ON COMMON KESTREL

# CHAPTER 3

## ECOTOXICOLOGICAL INVESTIGATIONS ON COMMON KESTREL

The present chapter concerns the ecotoxicological studies on Common kestrel (*Falco tinnunculus*).

### 3.1 State of the art, gaps and future perspectives on Common Krestel ecotoxicology

As previously described in the structure of the thesis, this chapter includes an introduction represented by a published review paper that investigates the gaps of knowledge on the toxicology of this raptor and the methods used or suggested to evaluate the ecotoxicological effects on Common Krestel individuals.

The review entitled “State of the art, gaps and future perspectives on Common Krestel ecotoxicology” is included in the present paragraph as pdf of the paper, published on Environmental Toxicology and Pharmacology by Giovanetti et al., 2023. The supplementary material of this paper is included in the present thesis as reported in the online version of the article (Section “supplementary material”).

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# State of the art, gaps and future perspectives on common kestrel ecotoxicology

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ABSTRACT

Anthropogenic activities have caused a steady decline of common kestrel (*Falco tinnunculus*) since the 1980 s. Effects, especially sublethal effects of contaminants, need to be investigated to ensure the conservation of this species. Data about countries, biological material, contaminants classes, and methodological approaches were collected from scientific publications to highlight gaps on common kestrel toxicology and ecotoxicology. We found that most studies have been conducted in Europe and in the field, underlining a lack of in vitro studies. The studies investigated mainly contaminant levels, while sublethal effects, evaluation of emerging contaminants and use of non-invasive or low-invasive samples were scarce. This work shows important gaps on toxicological status of the common kestrel, highlighting the importance of developing a non-lethal approach that combines responses at different levels of biological organization, as well as data on chemical contamination and on the environment in which the different populations inhabit.

## 1. Introduction

The common kestrel (*Falco tinnunculus*), also known as the Eurasian kestrel, is a small and diurnal raptor of the *Falconidae* family. It is distributed in Europe, Africa and, Asia (Cardozo et al., 2016), spreading across open lands such as grassland, shrubland, farmland (Casagrande et al., 2008) and urban centres (Charter et al., 2007). The *Falco tinnunculus* is considered a partially migratory species because it's totally migrant in cooler northern Europe and more resident in central and southern regions (Baltag et al., 2014; Holte et al., 2016). This species is characterized by a strong sexual dimorphism (Zampiga et al., 2008) with evident plumage and size differences (Fargallo et al., 2002; Piault et al., 2012). Generally, it's a solitary species but can be gregarious in favourable habitat (Ermolaev, 2016). Its nests are located in rock crevices, cliffs, buildings, and occasionally, in abandoned nests of other bird species; this species also exploits nest boxes (Anushiravani and Sepehri Roshan, 2017). In Europe, eggs laying occurs between mid-March and early June (Costantini and Dell'Omo, 2020), the incubation lasts about one month and the ledging period varies from 27 to 39 days (Kabeer et al., 2021). The common kestrel is a predator with a varied and variable diet, depending on the geographic region and, therefore, on the type of prey availability. For example, it feeds mainly on small mammals in northern Europe, such as voles and harvest mice (Laaksonen et al., 2008); otherwise, in southern regions, its diet is characterized by lizards and insects (Carrillo et al., 2017).

The global common kestrel population is around 4000,000 - 6500,000 mature specimens (BirdLife International, 2021) and about 19% of these individuals inhabit Europe (BirdLife International, 2016). The IUCN consider this species as "least concern" at the global level, although it is subject to moderate but steady decline since the 1980 s (BirdLife International, 2016). The European population size is estimated to be decreasing by about 25% in three generations (BirdLife International, 2015) and in some African countries the common kestrel population decreased by about 75–94% in the late 1960 s and early 2000 s (Thiollay, 2007).

The causes of the *Falco tinnunculus* decline are complex and multiple and are due to anthropogenic activities: agricultural intensification, pesticide use, habitat modification (e.g. sealed surfaces) (Grande et al., 2018), landscape simplification (e.g., proportion of arable land) (Butet et al., 2022), loss of nesting sites (Costantini and Dell'Omo, 2020), and even wind energy development (Strix, 2012). Among the different threats, the agricultural sector is the most important for the common kestrel decline (BirdLife International, 2016; Costantini et al., 2014). The plant protection products (e.g., insecticides, rodenticides, fungicides, and herbicides), widely used in this sector, can cause, for instance, alterations of nestling's body condition (Martínez-Padilla et al., 2017)

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and breeding success (Buck et al., 2020). Moreover, the effects of these substances can even be lethal (Hughes et al., 2013; Ruiz-Suárez et al., 2015). As an apex predator species, the common kestrel can accumulate higher concentration of contaminants than species at the lowest levels of the food web because of biomagnification processes (Yu et al., 2013).

In the last ten years, research on common kestrels has focused on contaminant accumulation (Jin et al., 2016; S. Kim et al., 2016; Luzardo et al., 2014; Roos et al., 2021), abundance (Butet et al., 2010; Gil-Tena et al., 2015; Teillard et al., 2014), behaviour (Abbasi et al., 2015; Schabacker et al., 2014) and reproductive performances (Costantini et al., 2014) in urban and agricultural habitats impacted by anthropogenic activities. At the same time, sublethal effects of toxic compounds have been poorly investigated (Bang et al., 2019; Buck et al., 2020; Shimshoni et al., 2012; Strong et al., 2015; Sumasgutner et al., 2018; Wemer et al., 2021).

The main aim of this paper was to review the available studies concerning the toxicology and ecotoxicology of the common kestrel to highlight any gaps and establish priorities for future investigation. In addition, the specific goals were to evaluate where the studies were conducted, the type of contaminants investigated, the considered life stages, the biological material tested, and the methodological approaches used.

## 2. Material and methods

A systematic quantitative approach (Pickering and Byrne, 2014), with some modifications, was adopted to conduct the research on common kestrel toxicology. Electronic bibliographic databases research was conducted on Scopus and Google Scholar and a time limit of eleven years (from 2010 until 2021) was adopted for the research. A group of keywords was established to identify relevant studies, where "*Falco tinnunculus*" was combined with the following terms: "toxic", "toxicology", "contaminants", "pesticides", "heavy metals", "sublethal effects" and "biomarkers". Only articles in English language and peer-reviewed were collected, while reviews were not included, although their references were checked to guarantee that no publications were missed. The references of selected articles and papers with one or more cited articles on *F. tinnunculus* were screened to assess whether they contained relevant studies to include in the present review. Book chapters, conference abstracts, reports, and other types of grey literature were not considered for this work.

Our search resulted in a total of 1226 studies, combining all the keywords and setting the parameters listed above. Articles regarding ecology, physiology, immunology, parasitology, toxicity of endogenous compounds and causes of admission to rescue centres were not selected, as long as the evaluated parameters were not correlated with contaminant exposure. Studies on physiological, biochemical/cellular, and behavioural variations in common kestrel of urban and agricultural areas variously exploited by human were included. The final selection comprised a total of 58 articles that met our criteria. The selected papers were examined in detail, recording the following information in an Excel database: year of publication, geographical location, life stage, sex, study type, biological material, contaminant classes, methodological approach and results of the paper.

If a work included multiple categories for any variable, all were considered in the final analyses and the definition "not specified" was assigned when a category information was missing.

The variable "studies type" was divided into four categories: "historical collection", "historical database", "field studies" and "laboratory studies". "Historical collection" and "historical database" indicated respectively the studies performed on biological material (such as feathers) kept in museums and datasets on poisoning events. "Field studies" included works conducted on wild birds, and "laboratory studies" identified exposure experiments in the laboratory. The "life stage" was divided into four categories: "eggs", "nestlings", "juveniles" and "adults". The variable "methodological approach" was divided into the following categories: accumulation, biochemical/cellular responses, dietary habits, body condition, behaviour, parasitology, poisoning, reproductive success and risk assessment. In particular, "accumulation" and "poisoning" papers evaluated the contaminant levels and poisoning events respectively. Biochemical/cellular responses" and "parasitology" studies included the molecular research and parasites presence, respectively. "Body condition" indicated the studies that calculated the body condition index and "reproductive success"; the latter referred to research on, for example, clutch size and hatching success. "Behaviour" and "dietary habits" described studies on this species abundance in a specific area and on dietary sources and trophic position. Concerning "risk assessment" research that evaluated, for instance, the Toxicity Exposure Ratio (TER) or the Hazard Quotient (HQ) were identified.

The results of each study were categorized with a symbol "+" when a study showed an effect and the symbol "-" when the authors reported no effect. For each study, the sample size, the result range or the mean value with standard deviation and the corresponding unit of measurement were recorded.

## 3. Results and discussion

The 58 studies selected on toxicology and ecotoxicology of the common kestrel, obtained from the bibliographical research spanning from 2010 to 2021, are reported below and divided by the recorded information: year of publication; geographical location; life stage and sex; study type; biological material; contaminant classes; methodological approach and results of the paper. Most of these studies (44) were conducted on multiple avian species, and only 14 considered just *F. tinnunculus*. The multispecies approach is often adopted to evaluate the levels or effects of different contaminants on avian species, which may have different feeding habits (diurnal or nocturnal), diet composition (e.g., insectivorous or carnivorous), or behaviour (e.g., breeder or resident) (Ma et al., 2021).

### 3.1. When and where the studies were performed

During the last eleven years, 58 papers were published with an average of five per year, and the major number of studies was published in 2014 (7) and 2021 (9) (Fig. 1).

The studies were conducted in all the countries where the species is distributed, in Eurasia and Africa, (Village, 1990). Specifically, most of the studies were carried out in Europe (40), followed by Asia (16) and Africa (2). The number of studies conducted in Europe can be related to the large number of European monitoring programmes that used birds of prey to investigate the impact of contaminants on the environment, were the common kestrel was one of the diurnal raptors most used as bioindicator (Gómez-Ramírez et al., 2014). Moreover, the high number of articles published by European researchers is also probably due to common kestrel population decline events (e.g., between 1980 and 2016) mainly related to agricultural practices (Roos et al., 2021).

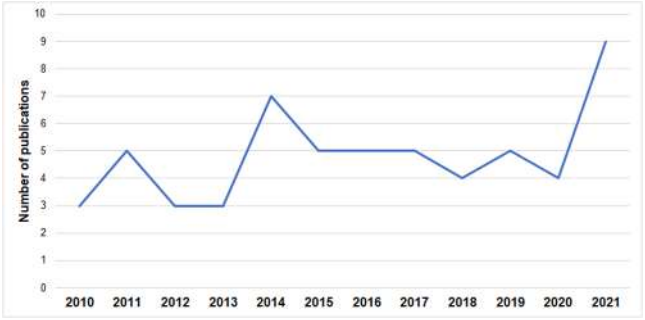


**Fig. 1.** Number of studies on common kestrel toxicology and ecotoxicology that met the criteria for inclusion in this review divided by year of publication.

Indeed, as reported by the European Environment Agency (2020), Europe is one of the continents with the most intensive agriculture and forestry activities. To confirm this, among the European countries, Spain (12) and France (6) were the ones with the highest number of publications. According to Eurostat (2021) these countries are part of the "big four" that strongly contribute to the EU's agricultural industry. It is maybe for this reason that in Spain and France, there was a greater interest in the evaluation of pesticides effects and habitat simplification on avian species such as common kestrel (Bouvier et al., 2011; Buck et al., 2020; Butet et al., 2010; Filippi-Codaccioni et al., 2010; Gil-Tena et al., 2015; Luzardo et al., 2014; Martínez-Padilla et al., 2017; Rial-Berriel et al., 2020, 2021; Ruiz-Suárez et al., 2014, 2015; Teillard et al., 2014; Valverde et al., 2020). On the other hand, despite Italy and Germany are included in the "big four," the number of publications in these countries was low: two and one, respectively. Four studies were published in United Kingdom (UK), probably because of the significant decline (−35%) that the Eurasian kestrel suffered in this country between 1995 and 2018 (Harris et al., 2020).

In general, however, the number of articles published by European researchers is still low considering the population decrease shown in many European countries, as published by Bird International (2021).

Regarding the Asian continent, most papers were conducted in Korea (6) and China (5). Abbasi et al. (2016) have reported a study's scarcity on the contaminant accumulation and toxicity of Asian birds. This finding, together with the growing and rapid industrialization of this continent, has prompted researchers to investigate accumulation and contaminant effects (Bang et al., 2019; Barghi et al., 2018; Jin et al., 2016; Kim and Oh, 2016; Kim et al., 2016; Ma et al., 2021; Nam and Lee, 2011; Shimshoni et al., 2012; Yin et al., 2018; Yu et al., 2013; Yu et al., 2011; Zhang et al., 2021).

The only two countries to have carried out studies on common kestrel toxicology in the African continent were Nigeria (1) and Egypt (1). The two papers were published recently, in 2021 and 2019 respectively. The only study evaluating the biopesticide effects in the last ten years was conducted on Nigerian common kestrel (Mullié et al., 2021).

This first analysis reveals that the research numbers at the global level are still too poor to guarantee a good monitoring and conservation of common kestrel.

### 3.2. Study type

Most of the studies of this review were conducted in the field (54), while research carried out on historical collections (1), historical databases (2), and in the laboratory (2) were only a few.

The "historical collection" research is an approach that allows to assess the geographical and temporal trend of the bird exposure to contaminants. Another positive aspect of this type of study is that the retrospective reference values obtained can be compared with current data of the same species living in the same geographical area (Movalli et al., 2017). Similarly, the studies on "historical databases" of poisoning can also be used for geographical and temporal trend evaluations and for identifying any flaws in the monitoring procedures related to toxic events. For example, Gil-Sánchez and collaborators (2021) showed that many animal groups, including the common kestrel, are over-represented in the national poisoning database while others are underrepresented. In general, this type of study offers many advantages even though such studies are still limited.

Regarding the "laboratory study," the number of publications was very low, probably because the common kestrel is strictly protected by different international agreements, limiting its use for scientific purposes.

Valverde and collaborators (2020) conducted an in vivo experiment on kestrel specimens recovered in a rescue center in southeastern Spain. The birds were exposed to bromadiolone, an anticoagulant rodenticide, to investigate its persistence at different carcass decomposition stages. It was possible to use these specimens for the experiments because they were intended for euthanasia due to severe wing injuries and all procedures complied the ethical standards. Their results suggest that the rodenticide persists until the first weeks after death under certain weather conditions; then the carcasses can be a source of wildlife poisoning. *In vivo* experiments are considered a valid approach for evaluating the contaminant effects, although they have obvious limitation due to the protected status of Eurasian kestrel and particularly for ethical issues. Therefore, in vivo testing to evaluate the contaminant effects is generally not desirable for different reasons. A valid alternative to in vivo studies could be in vitro research. This type of studies has many advantages such as animal-use reduction, control of chemical and physical environment, use of non or low-invasive matrices, and finally repeating experiment for several times. However, to this date, no study adopted an in vitro approach on common kestrel. Indeed, this type of approach is applied on other birds, reptiles and marine mammal species, showing to be a valuable tool to unravel interactions between contaminants and organisms and eventually also establish contaminant dose-response relationships (Bianchi et al., 2022; Crump et al., 2008; Hernández-García et al., 2014; Leena Mol et al., 2012; Oropesa et al., 2013;). Moreover, in some studies, a good correlation between in vivo and in vitro experiments was shown (Billiard et al., 2004; Head and Kennedy, 2010; Stadnicka-Michalak et al., 2015).

### 3.3. Life stages and sex

Concerning the life stages, fifteen studies (15) were conducted on adults, six (6) on nestlings, four (4) on eggs, and four (4) on juveniles. In thirty-seven (37) articles the specimen's life stage was not indicated or was not referred to the specific results. Most studies (55) did not specify the specimen's sex. Only 5 and 4 studies were conducted on males and females, respectively. In some articles of this research (8), age or sex were included as variables in regression models, even if only few works reported the specific result of the parameter measured related to stage of life or sex, as seen above.

In ornithology, it has been shown that the results of physiological parameters may differ depending on animal life stage and gender (Hao et al., 2021); it is therefore important to consider this aspect in ecotoxicological research too. For example, contaminant concentration can be higher in adult than juvenile birds due to the longer time exposure (Fritsch et al., 2019). Zhang and collaborators (2021) highlighted a higher contaminant (PCDD/Fs DL-PCBs and indicator of PCB) concentration in adult than juvenile common kestrels. On the contrary, the work of Grúz and collaborators (2019) did not show any significant differences between common kestrel age for the heavy metals (Cd, Cr, Pb and Hg) investigated. Concerning the sex variable, male and female specimens, having different hormonal levels, can show different responses in several parameters (Tartu et al., 2014). In this review only three papers evaluated differences between sex (Grúz et al., 2019; Hromada et al., 2011; Zhang et al., 2021). The results obtained by Hromada and collaborators (2011) on the Toxicity Exposure Ratio (TER) for rodenticides showed a difference between specimens' gender with a lower risk of poisoning in females than males *F. tinnuculus*. Both Zhang and collaborators (2021) and Grúz et al. (2019) showed no difference in the levels of contaminants between specimens of different sex. Taking into account the above results, life stage and sex in ecotoxicological research on birds remains an important point to be considered.

### 3.4. Biological materials

In the last ten years, as shown in Fig. 2a, 42 studies have been conducted on non-invasive or low-invasive samples of Eurasian kestrel: blood (6), eggs (5), feathers (10) and whole organism (21). The category "organism/population" includes papers on risk assessment, reproductive success, behaviour, and body condition that consider the specimen or the population in their entirety. Other 42 studies were conducted on

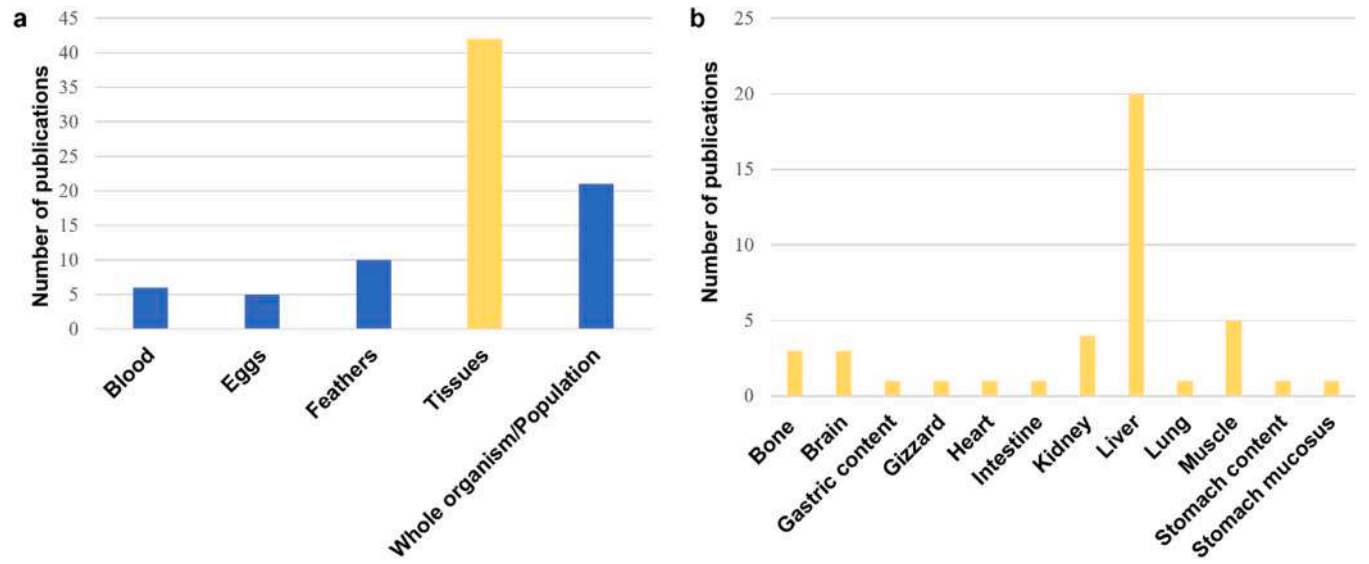


**Fig. 2.** Number of studies that met the criteria for inclusion in this review, divided by biological material (a) and the specific tissues used (b).

tissues obtained with a destructive approach (Fig. 2b): liver (20), muscle (5), kidney (4), bone (3) brain (3) and gastric content (1), gizzard (1), heart (1), intestine (1), lung (1), stomach content (1) and mucosus stomach (1). Of these, 7 papers have used more than one tissue.

The investigations that used a destructive sampling of the animals are carried out on stranded birds or birds to be euthanized in the rescue centers and provide good results on the degree of contamination of the animals (García-Fernández et al., 2013). However ethical and legal limitations are present and must be underlined (Yin et al., 2018). In addition, the use of samples from stranded or euthanized animals does not always guarantee a statistically significant number for an adequate assessment of the health status of the species. In the last years, there is a growing interest in the use of non-invasive (excreta, moulted feathers and eggshells) or low-invasive (blood and plucked feathers) biological materials. Among the various tissues available, most studies using a non-lethal approach focus on feathers (8 out of 10) because they are easy to collect and create a very low stress level on the animals. The feathers are also a valuable tissue for studying the effects of contamination by heavy metals, other non-essential elements (Manjula et al., 2015) and legacy POPs (together with plasma and preen oil). However, a Norwegian study showed that feathers are not suitable for the analysis of emerging contaminants (Løseth et al., 2019). Some authors (Abbasi et al., 2017; Manzano et al., 2021) also used this biological material to investigate the feeding ecology by stable isotopes analysis (δ13C and δ15N). In recent years the use of blood has increased to study the ecotoxicological effects of different contaminants on common kestrel (Berny et al., 2017; Martínez-Padilla et al., 2017; Rial-Berriel et al., 2020; Valverde et al., 2020; Wemer et al., 2021). Although it has proven to be an efficient material for the assessment of sublethal effects (Espín et al., 2016), not only in birds but in other protected species, the number of research studies using blood remains low.

In conclusion, when dealing with endangered species, the use of non and low-invasive, rather than invasive or destructive techniques, is preferable, as suggested by several authors (Casini et al., 2018; Fossi and Leonzio, 1994; Hopkins et al., 2005).

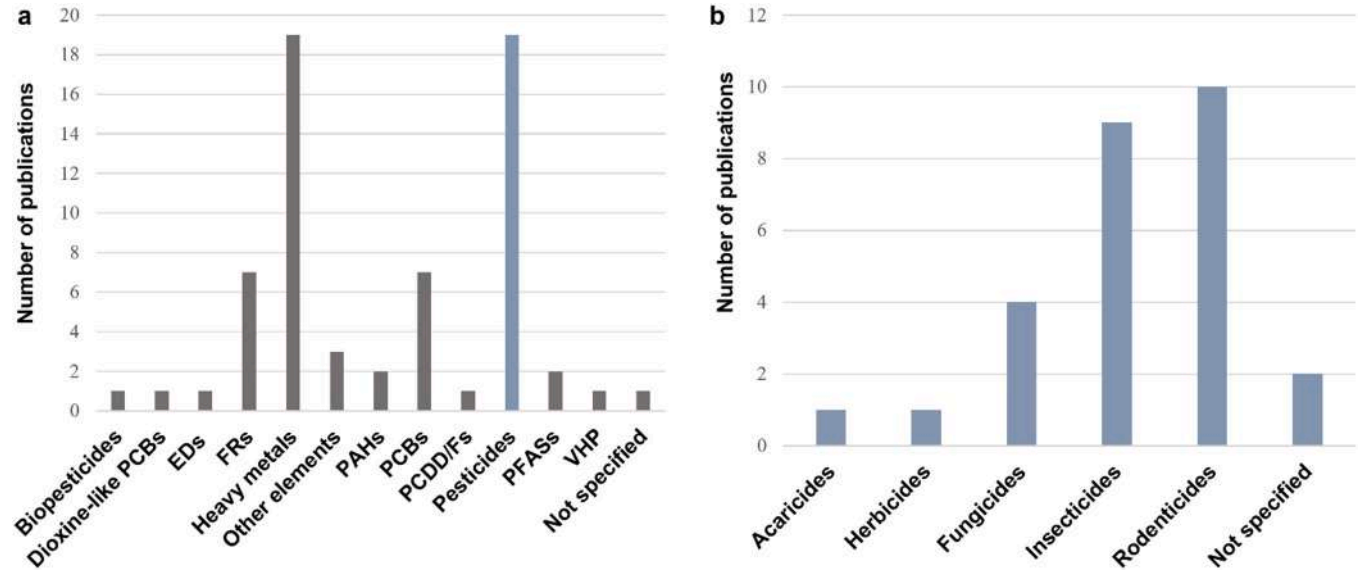


**Fig. 3.** Number of studies that met the criteria for inclusion in this review, divided by contaminant (a) and pesticide classes investigated (b). The "not specified" category referred to articles on poisoning without a contaminant investigation (a) or to papers without details on pesticide classes (b).

### 3.5. Contaminant classes

Regarding the classes of contaminants investigated in common kestrel, the Fig. 3a shows that most of the research studied the presence or the effects of pesticides (19) and heavy metals (18), followed by studies on flame retardant (7) and polychlorinated biphenyls (7). Among the 19 articles on pesticides, 10 focused on rodenticides class, followed by insecticides (9) and fungicides (5). The other types of pesticides were less investigated (Fig. 3b). In three of the 19 articles on pesticides, more than one class of pesticides were studied. In addition, eleven papers assessed effects or concentration of different substances belonging to the same class of pesticides. The most investigated chemical classes of pesticides were organophosphates (12), followed by coumarins (6), carbamates (5) and organochlorines (5). Regarding the studies on effects and abundance of heavy metals, the elements most investigated were Pb (13), Cd (12), Hg (9), Cu (8) and, Cr (6). The other heavy metals were less investigated (Appendix Table A1). Thirteen of the 19 papers on heavy metals measured more than one element. The large number of heavy metal studies is probably linked to the fact that birds, especially birds of prey, as top predators, tend to accumulate heavy metals (Abbasi et al., 2015), and they are then considered good bioindicators for the monitoring of toxic compounds (Grúz et al., 2019; Movalli et al., 2017; Espín et al., 2016).

Regarding pesticides, the strong interest in this topic is probably due to the fact that intensive agricultural activities require the increasing use of plant protection products (PPTs) for crops control. Since the 1970 s, it has emerged that many of these substances, some of which are no longer in use, have adverse effects on non-target species, including birds. Several authors studied the effects of PPTs on birds, suggesting that these compounds can accumulate in common kestrel (Bang et al., 2019; Barghi et al., 2018; Bouvier et al., 2011; Buck et al., 2020; Christensen et al., 2012; Hromada et al., 2011; Hughes et al., 2013; Kim et al., 2016; Luzardo et al., 2014 Martínez-Padilla et al., 2017; Rial-Berriel et al., 2020, 2021; Roos et al., 2021; Ruiz-Suárez et al., 2014, 2015; Schabacker et al., 2014; Shimshoni et al., 2012; Valverde et al., 2020; Yu et al., 2013). Sublethal effects, evaluated through acetylcholinesterase (AChE) (Bang et al., 2019; Shimshoni et al., 2012) and Protoporphyrin IX (Buck et al., 2020), reproductive success (Buck et al., 2020; Costantini et al., 2014; Sumasgutner et al., 2014, 2019), behaviour (Abbasi et al., 2015; Bouvier et al., 2011; Butet et al., 2010; Filippi-Codaccioni et al., 2010; Gil-Tena et al., 2015; Kitowski et al., 2016; Mullié et al., 2021; Roos et al., 2021; Schabacker et al., 2014; Sumasgutner et al., 2014, 2019; Teillard et al., 2014; Yaneva et al., 2020;), poisoning events (Gil-Sánchez et al., 2021; Hughes et al., 2013; Ruiz-Suárez et al., 2015) and risk assessment (Hromada et al., 2011; Yin et al., 2018; Yu et al., 2013; Zhang et al., 2021) were also investigated.

Various studies have also evaluated the effects of pesticides on common kestrel because some of these compounds, such as rodenticides and insecticides, have lethal effects on organisms that are the basis of the common kestrel diet. Feeding mainly of rodents, this species could suffer a secondary exposure to the substances mentioned above (Hughes et al., 2013). The reducing in the abundance of preys could also have an indirect effect by the generation of a series of cascading effects as diet modification, some food deficiency and, consequently, a reduction of the body condition, making the animal more susceptible to any pathologies.

Although fungicides and herbicides are among the most used pesticides in agriculture, to date they are poorly investigated in *F. tinnunculus*.

Few works monitored the emerging contaminants (ECs), such as perfluoroalkyl (PFASs) (Barghi et al., 2018; Eriksson et al., 2016), novel flame retardants (OPFRs, DPs, NBFRs, DBDPE, BTBPE, EHTBB, BEHTBP) (Abbasi et al., 2017; Jin et al., 2016; L. Yu et al., 2013) and UV filters (Molins-Delgado et al., 2017). In addition, no studies have been found on bisphenol replacement compounds such as BPS, BPF, BPAF, and chlorinated paraffins (CPs). An increase in studies on ECs is necessary, given that these compounds are widely spread in different environmental compartments (González -Rubio et al., 2021) and their effects on the common kestrel are poorly understood.

### 3.6. Method approaches

The majority of studies evaluated the accumulation of contaminants in common kestrel (40), followed by research on behaviour (13), dietary habits (7), biochemical/cellular responses (5), body condition (5), reproductive success (4) and risk assessment (4). The articles focused on parasitology (3) and poisoning (3) were lower (Fig. 4). Twenty-three articles of the 58 included in this review evaluated at least two different endpoints.

#### 3.6.1. Accumulation

The common kestrel, as an apex predator, can accumulate different classes of contaminants; for this reason, the number of works on the accumulation was the highest (40 out of 58). The results of all works on accumulation included in the review are summarized in the appendix (Table A2). Twenty-four of the works evaluated exclusively this parameter, while 16 articles combined this information with parameters aiming to evaluate their possible effects on the species. Only 9 out of 24 investigated the presence of multiple classes of contaminants (Abbasi et al., 2017; Barghi et al., 2018; Buck et al., 2020; Jin et al., 2016; Luzardo et al., 2014; Molins-Delgado et al., 2017; Rial-Berriel et al., 2020; L. Yu et al., 2013; Zhang et al., 2021). These results show the lack of an integrated approach that combines contaminant data and sublethal effects. Studies that use an integrated approach would provide more comprehensive information that can be used for the conservation of this species. Unfortunately, only a few authors adopted this method, for example Buck and collaborators (2020) evaluated OCs and PCBs levels, the effects on reproductive success (eggshell thickness), and the protoporphyrins IX concentration as a biochemical response in eggs. Similarly, Bang et al. (2019) also evaluated the accumulation of OP pesticides and sublethal effects by a biomarker test (AChE activity). However, the evaluation of several toxicological responses would have been desirable in order to obtain a more complete view of the studied contaminant effects.

In addition, most accumulation studies were conducted on destructive material (26 out of 40), such as liver, muscle, kidney, brain, etc. However, accumulation analysis can also be conducted on non-invasive or low-invasive material like excreta, blood and feathers. These tissues also enable the evaluation of the possible effects of toxic substances at different levels of biological organization.

#### 3.6.2. Biochemical and cellular responses

Among the papers that investigate the biochemical and cellular responses (5 articles), AChE activity (2), protoporphyrin IX (1) and carotenoid coloration (1) were assessed. One paper only evaluated more than one parameter (complement system activity; haptoglobin concentration, Hp; natural antibodies activity, Nab; total glutathione, tGSH; ratio glutathione:glutathione disulphide, GSH:GSSG) (Table 1). Both the studies on AChE highlighted basal values of the specimen (8–12 $\pm$ 1.77 μmol/min/g), but the activity values of exposed animals are not showed or determined. Buck and collaborators (2020) evaluated the concentration of protoporphyrins IX (a reddish-brown pigment in avian eggshells) as a biomarker of exposure to OCs showing a protoporphyrins concentration reduction with the increasing hexachlorobenzene level in the egg content. Although many OCs have been banned since the 1970 s, the results of this paper suggest continuing to monitor the presence of these substances in the environment in order to assess the effects of regulatory actions. The effects of urbanisation on common kestrel nestlings were also investigated by biochemical (Wemer et al., 2021) methods and skin yellowness measurements (Sumasgutner et al., 2018). Wemer et al. (2021) found that kestrel nestlings present in more urbanized areas had lower hemolysis, while none of the other immunotoxicity (NAb and Hp concentration) and oxidative stress (tGSH and

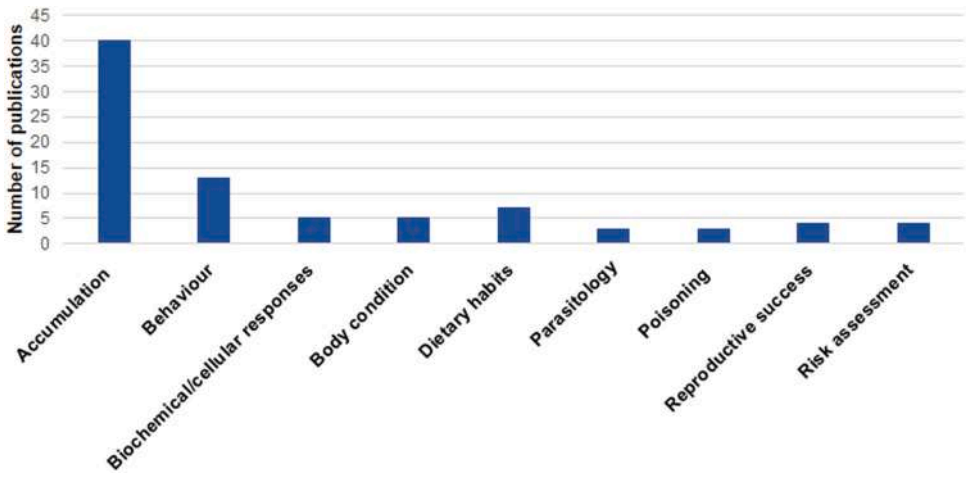


**Fig. 4.** Number of studies that met the criteria for inclusion in this review, divided by methodological approaches adopted.

**Table 1**
Studies that met the criteria for inclusion in this review, divided by biochemical/cellular parameters evaluated. The indicated studies did not specify the animal's sex, and all were conducted in the field. The abbreviation ns means "not specified", the symbols "+ " and "-" indicate an effect or no effect, respectively. nd indicates "not detected" and asterisk (*) refers to indicative results extrapolated from the graphs.

| Life stage | Biological material | Contaminant | Human exploited environments | Methodological approach | Sample size | Results (mean ±sd; mean; min-max) | | Unit of Measurement | Reference, year |
|---|---|---|---|---|---|---|---|---|---|
| **Nestlings** | Blood | *ns* | Urban | tGSH | 143 | - | | | Wemer et al. (2021) |
| | | | | GSH:GSSG | | - | | | |
| | | | | Complement system activity | 69 | + | *ns* | *ns* | |
| | | | | NAb activity | | - | | | |
| | | | | [Hp] | | - | | | |
| **Eggs** | Egg | HCB | | Protoporphyrin IX | 40 | + | 0–120 * | nmol/g d.w. | Buck et al. (2020) |
| ***ns*** | Brain | No pesticide exposure | | AChE activity | 5 | | 8.66 ± 1.04 | µmol/min/g tissue | Bang et al. (2019) |
| **Nestlings** | Animal | *ns* | Urban | Carotenoid coloration | 154 | + | -0.6 – 0.4 * | | Sumasgutner et al. (2018) |
| ***ns*** | Brain | Insecticide *ns* | | AChE activity | | - | *nd* | | Shimshoni et al. (2012) |
| | | No pesticide exposure | | | 21 | | 12.4 ± 2.5 | µmol/min/g tissue | |

**Table 2**
Studies that met the criteria for inclusion in this review, divided by dietary habits parameters evaluated. The animal's sex was not specified, and all the indicated studies were conducted in the field. The abbreviation ns means "not specified", the symbols "+ " and "-" indicate an effect or no effect, respectively.

| Life stage | Biological material | **Contaminant** | Methodological approach | Sample size | Results (mean±sd; mean; min-max) | | Unit of measurement | Reference, year |
|---|---|---|---|---|---|---|---|---|
| **Adults** | Feathers | Heavy metals and other elements | Dietary source (δ13C) | 37 | | -24.06 - – 19.29 | ‰ | Manzano et al. (2021) |
| | | | Trophic position (δ15N) | | + | 6.27–15.25 | | |
| **Adults** | Liver | PCBs, DDts, CHLs, CBz and PFASs | Trophic position (δ15N) | 4 | + | ns | ‰ | Barghi et al. (2018) |
| ***ns*** | Feathers | PBDEs, HBCDDs, BTBPE, BEHTBP and EH-TBB | Dietary source (δ13C) | 4 | - | –20.4 ± 4.8 | ‰ | Abbasi et al. (2017) |
| **Eggs** | Egg | BP1, BP3, 4HB, 4 DHB, ODPABA, OC and UVP | Trophic position (δ15N) | 10 | - | + 8.3 ± 2.1 | ‰ | Molins-Delgado et al. (2017) |
| | | | Trophic position (δ15N) | | - | 8.58–11.45 | | |
| **Eggs** | Egg | PFASs | Dietary source (δ13C) | 40 | | -28.2 | ‰ | Eriksson et al. (2016) |
| | | | Trophic position (δ15N) | | | 5.3 | | |
| **Adults** | Liver | PBDEs, BEHTBP, DBDPE, BTBPE and DP | Trophic position (δ15N) | 4 | + | 7.0–9.4 | ‰ | Jin et al. (2016) |
| ***ns*** | Muscle | PBDEs | Dietary source (δ13C) | 23 | | -18.4 ± 1.7 | ‰ | Yu et al. (2011) |
| | | | Trophic position (δ15N) | | | 7.2 ± 1.2 | | |

GSH:GSSG) parameters were altered. Sumasgutner and collaborators (2018) showed a reduction in carotenoid coloration in nestlings located in the city centre of Vienna. Further studies are needed to better enhance the relation between landscape ecology and biochemical and cellular responses. From this review, it emerged that biomarkers are scarcely used as predictive methodology of common kestrel exposure to contaminants. Further studies should also include the use of a set of biomarkers capable of providing different toxicological responses, such as neurotoxicity, oxidative stress, immunotoxicity, and genotoxicity.

### 3.6.3. Dietary habits

Seven articles investigated the common kestrel dietary habits related to contaminants exposure (Table 2). These aspects are usually examined by stable isotope analyses of carbon and nitrogen (δ13C and δ15N) because they permit to define organisms' dietary sources and trophic position (Yu et al., 2011) and their relation with the contamination (Abbasi et al., 2017). In particular, δ13C reflects the carbon source, while the δ15N can indicate the trophic level because its values enrich by 2–5% per trophic level (Eriksson et al., 2016). These parameters permit to investigate how the diet can increase or influence the exposure, accumulation, and biomagnification of a given contaminant (Jin et al., 2016; Molins-Delgado et al., 2017). This analysis can be carried out on different tissues (such as feathers, eggs, blood), but different information is obtained according to the used tissue. The isotopic composition of unhatched eggs reflects the isotopic signature of the mother birds (Molins-Delgado et al., 2017); the composition of feathers represents the diet habits during the moulting period (Manzano et al., 2021), whereas blood components reflect the trophic strategy during days or weeks before blood collection (Cherel et al., 2014; Kurle et al., 2013).

### 3.6.4. Body condition

A total of 5 papers evaluated the common kestrel body condition (Costantini et al., 2014; Martínez-Padilla et al., 2017; Sumasgutner et al., 2018, 2019; Wemer et al., 2021) (Table A3 in appendix). Wemer et al. (2021) found a negative correlation between body condition and urban gradient, and they assumed that the lack of suitable prey determined this condition. On the contrary, Sumasgutner and collaborators (2018) did not identify any relation between body condition and the urban environment. Regarding agricultural environments, nestlings from intensively cultivated areas showed worse body condition than those from grassland lands (Costantini et al., 2014). Sumasgutner et al. (2019) found no variation in the common kestrel adults from more or less exploited agricultural areas. Body condition was low in nestlings from two areas from central of Spain with residues of the rodenticide Bromadiolone (Martínez-Padilla et al., 2017). The body condition is considered an indicator of individual's health or quality (Peig and Green, 2009; Labocha et al., 2014). It is routinely used in avian studies and can include different morphological and physiological metrics that represent the nutritional, immune, and hormonal state of a specimen (Frauendorf et al., 2021). One of the most used body condition index is the body mass of an individual corrected to its size or age, and it is commonly applied to quantify energy reserves in birds (Costantini et al., 2009). The articles included in this review evaluated the body condition using morphological metrics and applying validated methods (García--Berthou, 2001; Green, 2001; Roulin et al., 2007). Considering all the results obtained from the above studies, it would be interesting to proceed with other studies that evaluate the relationship among physiological parameters, levels of contaminants and body condition index.

### 3.6.5. Behaviour

Eight (8) of the 13 studies on behaviour (Table 3) examined the common kestrel abundance and the occurrence frequency, four (4) investigated the feeding behaviours, and one (1) the timing of breeding. The studies included in this review show that habitat simplification and the potential presence of environmental contaminants can affect the common kestrel behaviour. From a research carried out in the UK (Roos et al., 2021), which combined historical data on Second Generation Anticoagulant Rodenticides (SGARs) levels with data on kestrel abundance (from 1997 to 2012), emerged that its presence decreased with the rodenticide's concentration increase in the same considered year. Gil-Tena et al. (2015) showed that the composition of the agricultural landscape could influence farmland bird diversity. This study displayed that the common kestrel, preferring areas with shrubs and hedges, was less present in more impacted agricultural areas, such as those cultivated with cereals, rotational grassland, or artificial lends. Similarly, Butet et al. (2010) showed a decrease, although not significant, in the abundance of the kestrel in western France, where grasslands, hedgerows, and wooded area decline are present. Another author highlighted that the kestrel could be exposed to pesticides even in non-agricultural grassland scenarios, such as golf courses, due to its high frequency in this type of landscape (Schabacker et al., 2014). The urban environment, like agriculture one, can also cause behavioural changes on birds (Møller and Ibáñez-Álamo, 2012), including common kestrel. The urban environment facilitates the permanence of the species as there is a greater availability of nesting sites; this may represent an ecological trap, as the species is induced to eat small birds instead of rodents, modifying its diet (Sumasgutner et al., 2014). Mulliè et al. (2021) found that individuals who ate grasshoppers treated with biopesticides (used to deal with entomopatogenic infestation by *Metarhizium acridum*) were subjected to a variation in food choice, preferring larger female locusts over smaller male ones. Up to date, no studies integrated behavioural results with

**Table 3**
Studies that met the criteria for inclusion in this review, divided by behavioural parameters evaluated. The animal's sex was not specified, and all the indicated studies were conducted in the field. The abbreviation ns means "not specified", the symbols "+ " and "-" indicate an effect or no effect, respectively.

| Life stage | Biological material | **Contaminant** | Human exploited environments | Methodological approach | Sample size | **Results** | Unit of measurement | Reference, year |
|---|---|---|---|---|---|---|---|---|
| ***ns*** | Animal | *Metarhizium acridum* | | Feeding behaviour | 40 | + *ns* | | Mullie´et al. 2021 |
| ***ns*** | Animal | Rodenticide *ns* | | Abundance | | + *ns* | | Roos et al. (2021) |
| ***ns*** | Animal | | Agricultural | Abundance | | - | | Yaneva et al. (2020) |
| **Adults** | Animal | | | Timing of breeding | 448 | - | | Sumasgutner et al. (2019) |
| **Adults** | Animal | Heavy metals | | Feeding behaviour | 3 | - | | Kitowski et al. (2016) |
| ***ns*** | Animal | Heavy metals | | Feeding behaviour | 5 | + *ns* | | Abbasi et al. (2015) |
| ***ns*** | Animal | | Agricultural | Abundance | | + *ns* | | Gil-Tena et al. (2015) |
| ***ns*** | Animal | Pesticide *ns* | Non-agricultural (golf course) | Frequency of occurrence | | + 55 | % | Schabacker et al. (2014) |
| **Nestlings** | Animal | | Urban | Feeding behaviour | 763 Nests | + *ns* | | Sumasgutner et al. (2014) |
| ***ns*** | Animal | | Agricultural | Abundance | | - | | Teillard et al. (2014) |
| ***ns*** | Animal | Insecticides *ns* Fungicides *ns* | | Frequency of occurrence | | + 20 | % | Bouvier et al. (2011) |
| ***ns*** | Animal | | Agricultural | Abundance | | + *ns* | | Butet et al. (2010) |
| ***ns*** | Animal | | Agricultural | Abundance | | - | | Filippi-Codaccioni et al. (2010) |

molecular, biochemical, cellular and physiological investigations to obtain information on the general health status of the species. Indeed, further studies that investigate effects at different biological levels of organization are needed for the conservation of the species.

### 3.6.6. Parasitology

The presence of parasites was evaluated in three studies of the review (Table A5 of appendix). Wemer et al. (2021) and Sumasgutner et al. (2018) conducted their research on kestrel nestlings from the city of Vienna. Both studies did not show any infection of ectoparasite *Carnus hemapterus* related to urban gradient. However, ectoparassites were higher in junior siblings than their senior siblings (Wemer et al., 2021) and in earlier nests of the season (Sumasgutner et al., 2018). A result similar to the latter was obtained again by Sumasgutner and collaborators (2014), where a seasonal decline of parasite infection intensity in kestrel chicks was found. Sumasgutner and collaborators (2018) also showed that parasite infection was higherin chicks with less intense skin yellowness, probably because the immune system is activated to fight the parasite infection. Therefore, it requires more circulating carotenoids that cannot be used for pigmentation.

On the contrary, Sumasgutner et al. (2019) evaluated a blood parasite infection (*Haemoproteus, Plasmodium, and Leucocytozoon*) of a common kestrel migratory population from agricultural fields in western Finland. The parasite infection was used as a parameter of individual quality (together with body condition). It was not correlated with landscape homogenization linked to agricultural intensification.

As shown, there are very few studies on parasitology related to urban and rural gradients and research that investigate the interplay between the type of environment, level of contaminants, and individual quality. In addition, more studies on the cause of the parasite's presence are necessary to understand all interactions with physiological signals (i.e., immunotoxicity and oxidative stress biomarkers).

### 3.6.7. Poisoning

Poisoning events were evaluated in three articles (Table a4 in appendix). Gil-Sánchez et al. (2021) compared the results of their field observation with the national poisoning database (from 1990 to 2015) of Spain. They showed an over-representation of the common kestrel in the intentionally poisoned database. Ruiz-Suárez and collaborators (2015) found that in the Canary Islands, the intentional poisoning of the common kestrel was still frequent from 2010 to 2013. In fact, out of 17 kestrels analysed, pesticides poisoned 15 specimens. These results show the relevance of pesticide poisoning as cause of death of common kestrels from the Canary Islands. It is important to note that among the pesticides found in carcasses, two (Carbofuran and Aldicarb) were already banned in the EU during the years of research. Finally, Hughes et al. (2013) highlighted that up to 23 kestrels, 41% were exposed to rodenticides. These results contrast with the Wildlife Incident Investigation Scheme (WIIS) Scotland, where among the causes of death of kestrels poisoning was 0%. From these results it emerged that this kind of study is still helpful for wildlife conservation.

### 3.6.8. Reproductive success

Most of the parameters investigated on reproductive success were fledging success (4) and hatching success (3), followed by clutch size (2), number of fledglings (2), nestling survival (1), egg volume (1), and eggshell thickness (1) (Table 4). The majority of these studies highlighted that urban and agricultural environments could affect the breeding success of common kestrel. The nests failure of urban kestrels in the city of Vienna was caused by predation or desertion of the nests and it occurred during eggs incubation. Moreover, male chicks were more subjected to death, and eggs hatched later than the average of the specie (Sumasgutner et al., 2014). Costantini and collaborators (2014) recorded a delayed egg-laying and a poor condition of offspring in specimens from the Italian intensive agriculture areas, and no effects on reproductive parameters. On the contrary, Sumasgutner et al. (2019) showed that the landscape homogenization due to agricultural practices intensification could affect the fledging success. Finally, Buck and collaborators (2020) investigated the levels of PBCs and OCs in specimens from the island of Tenerife, showing a reduction in eggshell thickness probably due to the presence of p,p'-DDE in the surface of active and abandoned croplands in a 200 m-radius around the nest and with proximity to urban areas. These data demonstrate the importance to evaluate the different aspects of the reproductive success for a more accurate and effective conservation of the species. A very innovative approach would be to link these parameters with the evaluation of biochemical, cellular and tissue responses of each organism to prevent long-term effects on the individual and the general population.

### 3.6.9. Risk assessment

The risk assessment was carried out in 4 articles (Table 5), and different methods were applied (Toxic equivalency, TEQ; Hazard quotient, HQ; Bioaccumulation factor, BAF; Toxicity-Exposure-Ratio, TER). These studies showed that contaminants such as PCDD/Fs, PCBs, Dioxin-like PCBs, PBDEs, and DDTs represent a potentially high hazard for common kestrel (Yin et al., 2018; L. Yu et al., 2013; Zhang et al., 2021).

**Table 4**
Studies that met the criteria for inclusion in this review, divided by reproductive success parameters evaluated. The animal's sex was not specified, and all the indicated studies were conducted in the field. The abbreviation ns means "not specified", the symbols "+ " and "-" indicate an effect or no effect, respectively. Asterisk (*) refers to indicative results extrapolated from the graphs.

| Life stage | Biological material | **Contaminant** | Human exploited environments | Methodological approach | Sample size | Results (mean ±sd; min-max) | Unit of Measurement | Reference, year |
|---|---|---|---|---|---|---|---|---|
| **Eggs** | Egg | DDTs, PCB | | Eggshell thickness | 40 | + 0.14–0.22 * | mm | Buck et al. (2020) |
| | | | | Fledging success | | - | | |
| | | | | Hatching success | | - | | |
| **Nestlings** | Animal | | Agricultural | Fledging success | | + | | Sumasgutner et al. (2019) |
| ***ns*** | Egg | | Urban | Clutch size | 157 broods | - | | Sumasgutner et al. (2014) |
| | | | | Hatching success | | + | | |
| **Nestlings** | Animal | | | Number of fledglings | | + | | |
| | | | | Fledging success | | - | | |
| **Eggs** | Egg | | Agricultural | Clutch size | 109 | - 5.39 ± 0.15 | n | Costantini et al. (2014) |
| | | | | Egg volume | 341 | - 56.62 ± 0.28 | cm3 | |
| | | | | Hatching success | 107 | - 81.32 ± 5.04 | % | |
| **Nestlings** | Animal | | | Number of fledglings | 113 | 4.25 ± 0.28 | n | |
| | | | | Fledging success | 106 | - 96.56 ± 1.67 | % | |

**Table 5**
Studies that met the criteria for inclusion in this review, divided by risk assessment parameters evaluated. The abbreviation ns means "not specified", the symbols "+ " and "-" indicate an effect or no effect, respectively. Asterisk (*) refers to indicative results extrapolated from the graphs.

| Life stage | **Sex** | Study type | Biological material | **Contaminant** | Methodological approach | Sample size | Results (min-max) | Unit of Measurement | Reference, year |
|---|---|---|---|---|---|---|---|---|---|
| **Juveniles** | F | Field | Muscle | PCDD/Fs, PCBs and | TEQ | 25 | + 0.03–0.6 * | pg/g l.w. | Zhang et al. |
| | M | | | Dioxin-like PCBs | | | + 0.03–0.09 * | ng/g w.w. | (2021) |
| **Adults** | F | | | | | | + 0.01–0.5 * | ng/g w.w. | |
| | M | | | | | | + 0.05–0.8 * | ng/g w.w. | |
| ***ns*** | *ns* | Field | Animal | PBDEs | HQ | 17 | + > 10 | | Yin et al. (2018) |
| ***ns*** | *ns* | Field | Liver | DDTs | HQ | 23 | + 0.15 | | Yu et al. (2013) |
| | | | | PCBs | | | - 0.042 | | |
| | | | | PBDEs | | | - 0.067 | | |
| **Adults** | *ns* | Laboratory | Animal | Brodifacoum | BAF | 2 | - 0.05 | | Hromada et al. |
| | *ns* | | | Warfarin | BAF | | - 0.04 | | (2011) |
| | M | | | Brodifacoum | TER | | - 24.76 | | |
| | M | | | Warfarin | TER | | - 571.3 | | |
| | F | | | Brodifacoum | TER | | - 45.61 | | |
| | F | | | Warfarin | TER | | - 4285.7 | | |

On the other hand, Hromada and collaborators (2011) showed a low risk for kestrel linked to secondary exposure to rodenticides.

The results of these works show that further investigations are necessary on the risk assessment, considering not only the presence of contaminants but also the biochemical responses. Indeed, to identify the different risks to which *Falco tinnunculus* may be exposed and, therefore, to obtain useful information for decision-making, a proper risk assessment procedure should consider as much endpoints as possible.

The results of these works show that further investigations are necessary on the risk assessment, considering not only the presence of contaminants but also their effects by biochemical responses. Indeed, to identify the different risks to which *Falco tinnunculus* may be exposed and, therefore, to obtain useful information for decision-making, a proper risk assessment procedure should consider as much endpoints as possible. Moreover, in this procedure, should be considered also multiple stressors, such as contamination, climate change, poisoning, and habitat reduction, that affect not only the individual but also the population of common kestrel. This is important also to predict the combined effects of stress factors on common kestrel.

## 4. Conclusions

This review underlines some important gaps in the knowledge of the toxicological status of the common kestrel and from the gaps some suggestions for future research and monitoring studies arise. The first gap concerns the fact that papers on the toxicology of this species are in limited number and concentrated in some specific geographical areas, while in some area's information are almost completely lacking. There is then a need for increase the monitoring activity in general and particularly in some areas such as Africa and Asia continents and in some European countries like Italy and Germany.

A second important point to be considered is that non-lethal monitoring approaches should be developed and conducted, through in vitro and in vivo analysis using non or low-invasive sampling and monitoring methods. This approach allows to investigate alive and healthy animals and to have a realistic picture of the toxicological status of wild populations. Not only legislative or ethical reasons support this choice, but also scientific, including the potential for monitoring high numbers of specimens for repeating sampling several times over the time even in the same specimen or population. *In vitro* testing can help unravelling mechanisms that are at the base of the toxicology of the common kestrel and identifying compounds or mixtures to which the species is particularly sensitive. Excellent materials are blood, excreta, and feathers. Monitoring contamination in dead animals can give only a partial information and do not help in the study of sublethal and long-term toxicological effects.

A further gap of knowledge concerns the lack of information on the presence and effects of emerging contaminants in the common kestrel, suggesting the specific need to implement in vitro and in vivo studies on this subject.

It was also highlighted the lack of knowledge and therefore the need to significantly increase the studies relating to the sublethal effects of contaminants in this species. Studies on different effects such as genotoxicity, neurotoxicity, on the immune system and estrogenicity, should be conducted in a non-lethal way for the species.

But what is configured as the fundamental aspect and the most important gap to be bridged regarding the toxicology of this species and of avian species in general, concerns the development and application of a multitasking approach. It would allow to integrate information at different levels of biological organization, from the molecular and cellular and in general sub individual level, to the individual and population, including data on chemical contamination and on the environment in which the different populations live. As we have seen in this paper, to date the studies have essentially focused on specific aspects of toxicology, while an integrated approach could permit to have a complete picture of the health status of this species in the different environments. Risk assessment should be conducted considering the whole set of information and not just a few endpoints.

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## CRediT authorship contribution statement

**Silvia Casini:** Conceptualization, Supervision, Validation, Writing – review & editing; **Ilaria Caliani:** Conceptualization, Methodology, Writing – original draft, Supervision; **Laura Giovanetti:** Methodology, Investigation, Formal analysis, Data curation, Writing – original draft; **Tommaso Campani:** Methodology, Data curation.

## Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

## Data Availability

The data that has been used is confidential.

## Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.etap.2023.104237.

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## 3.2. Aim

Environment is negatively affected by anthropogenic activities. Urbanization, industrialization, and agricultural activities are causing profound changes of natural environments, modifying wildlife habitats and releasing a wide range of contaminants into the environment. These stressors can cause changes and potentially discomforts on animal's physiology and behaviour, birds included. The Common Kestrel (*Falco tinnunculus*) is a small diurnal raptor that lives both in urban and agricultural environments and, despite its classification as "Least Concern" in the IUCN Red List, it showed a modest but constant decline since the 1980s. It has been shown that urban environments are not optimal for Common Kestrels as well as agricultural habitats.

This species can be affected by multiple stressors therefore it is of fundamental importance to apply a multi-biomarker approach that allows to evaluate different biological and toxicological responses of Common Kestrel individuals in response to stressors such as habitat changes and contamination.

The literature showed important gaps in the knowledge of the toxicological status of the common kestrel and also in how multiple environmental changes can influence its physiology. Based on our knowledge, most of the field studies investigated the accumulation of different contaminants, while few papers focused on the sub-lethal effects of environmental contamination.

Moreover, it is important to underline that very few studies adopted a multi-biomarker approach which allows to evaluate different toxicological responses but also to investigate possible physiological variation induced by habitat modification. Still, what is really missing is a combined approach that integrates information at the sub individual level with data on chemical contamination and on the environment in which the different populations live. This approach could permit to gather information on the health status of the species in the different environments and on how the populations respond/adapt to different situations.

Another research gap is constituted by the lack of studies on non-lethal monitoring approaches that could be developed and conducted, through *in vitro* and *in vivo* analysis using non or low-invasive sampling. *In vitro* testing can help unravelling mechanisms that are at the base of the toxicology of the Common Kestrel and identify compounds or mixtures to which the species is particularly sensitive to. Information is also lacking on the presence and effects on Common Kestrel of emerging contaminants but also on pesticides such as widely used herbicides and fungicides. This suggests the need to implement *in vitro* and *in vivo* studies on this species.

In light of the above aspects, this part of the work of thesis aims to evaluate the ecotoxicological status and the physiological responses of Common Kestrel populations from areas with different anthropic pressures by applying a blood-based multi-biomarker approach combined with contaminant analysis.

To achieve this goal, specific aims of the study will be:

- To develop on blood sensitive and reliable biomarkers of genotoxicity and immunotoxicity able to evaluate important toxicological responses, very poorly investigated in this species up to now. To extend a consolidated panel of biomarkers, enriching it with markers that give information on DNA damage (comet assay) and innate immune response (respiratory burst), the latter strongly linked with other important physiological parameters.

- To validate the developed biomarker of genotoxicity through *in vitro* studies. To evaluate, *in vitro*, the genotoxicity of an herbicide and a fungicide widely used in the agriculture. To get information on the toxicology of the two tested compounds.

- To apply a multi-biomaker and combined approach on Common Kestrel populations inhabiting urban, rural and natural habitats. To compare the oxidative status (total glutathione tGSH, reduced and oxidised glutathione GSH and GSSG, the ratio GSH:GSSG, the non-enzymatic antioxidant capacity), immunological function (respiratory burst, differential white blood cells (WBCs) count, complement system activity, bacterial killing assay), genotoxicity (comet and erythrocytes nuclear abnormalities (ENA) assays), and neurotoxicity effects (butyrylcholinesterase and carboxylesterase (BChE and CaE) activities) of the common kestrel populations object of the study. To integrate blood markers information with contaminant (heavy metals, PCBs, OCPs, PBDEs and PCDD/Fs) chemical analysis conducted on non-lethal biological material (abandoned eggs and feathers) to investigate how the different environments can influence birds physiology and have a more complete picture of their state of health.

### 3.3. Development of comet assay and respiratory burst test on Common Kestrel

Based on our knowledge, there are several gaps concerning the toxicology of Common Kestrel. These include a lack of toxicological studies of some pesticides, such as herbicides and fungicides, and a lack of studies on the genotoxicity of these compounds on this species.

The main aim of the studies reported in this paragraph was to develop sensitive and reliable biomarkers not yet applied on Common Kestrel to evaluate genotoxic and immunotoxic effects. To

achieve this goal blood samples from individuals of Common Kestrel from a LIPU centre (Italian League of Bird Protection) (Livorno) and a Raptors rescue centre (Barberino del Mugello) were used. Blood sampling was conducted by a veterinarian from the jugular vein of individuals using a 1 mL syringe. Whole blood samples were transferred in a vacutainer (MICROMED®, Italy) containing lithium-heparin as anticoagulant and stored at 4° C for the transport to laboratory. Once arrived in the laboratory, the blood was processed for the tests to be developed.

*Comet assay*

The comet assay allows to detect alterations (e.g. breakages at one or both filaments, alkaline labile sites, repair sites, etc.) on DNA of eukaryotic cell, blood cells included. Briefly, cells are embedded in an agarose mat and lysates to diffuse the nuclear component, immersed in a strongly basic buffer (pH=13) and subjected to electrophoresis, during which DNA molecules with alterations migrate forming a comet. Comets are then observed with fluorescence microscopy and any damage evaluated with a software that quantifies the percentage of DNA in the tail of the comets. A higher percentage of DNA in the tail indicate a greater damage.

The comet assay on the Kestrel was developed modifying the method of Caliani et. al (2014). Tests were carried out on fresh and frozen blood. The blood sampling of the nestlings takes a long time as the various hatches follow one another within a few days and therefore the sampling effort is enclosed in a few days. This would not allow to carry out the comet assay in the field; the cryopreservation of the blood would have allowed the test to be carried out also in the days following the sampling and therefore would give the possibility to obtain important information on possible genotoxic effects in the avian species.

*Comet assay with fresh blood of Common Krestel*

Tests were conducted on fresh blood (3 samples for each condition) and, keeping the Caliani et al. (2014) method as a working basis, some of the main parameters of the comet assay were modified and tested: the dilution of samples in Phosphate Buffered Saline (PBS) and the timing of the electrophoretic run. During these tests, the following parameters were adopted:

- Dilutions: One dilution (1:100), or two dilutions (1:100 and 1:20), two dilutions (1:1000 and 1:4);
- Electrophoresis time: 10 minutes or 15 minutes at 16 V and 250 mA.

The different laboratory conditions are summarized in the Table 3.1.

**Table 3.1.** Different parameters modified for the development of the comet assay in Common Kestrel. Each letter, after represented in the graph, indicates a different condition tested.

| | **LABORATORY CONDITION** |
|---|---|
| **A** | 1 dilution (1:100) - 0.5% LMA - 10 min of electrophoresis |
| **B** | 1 dilution (1:100) - 0.5% LMA - 15 min of electrophoresis |
| **C** | 2 dilutions (1:100 and 1:20) - 0.5% LMA - 10 min of electrophoresis |
| **D** | 2 dilutions (1:100 and 1:20) - 0.5% LMA - 15 min of electrophoresis |
| **E** | 2 dilutions (1:1000 and 1:4) - 0.5% LMA - 10 min of electrophoresis |
| **F** | 2 dilutions (1:1000 and 1:4) - 0.5% LMA - 15 min of electrophoresis |

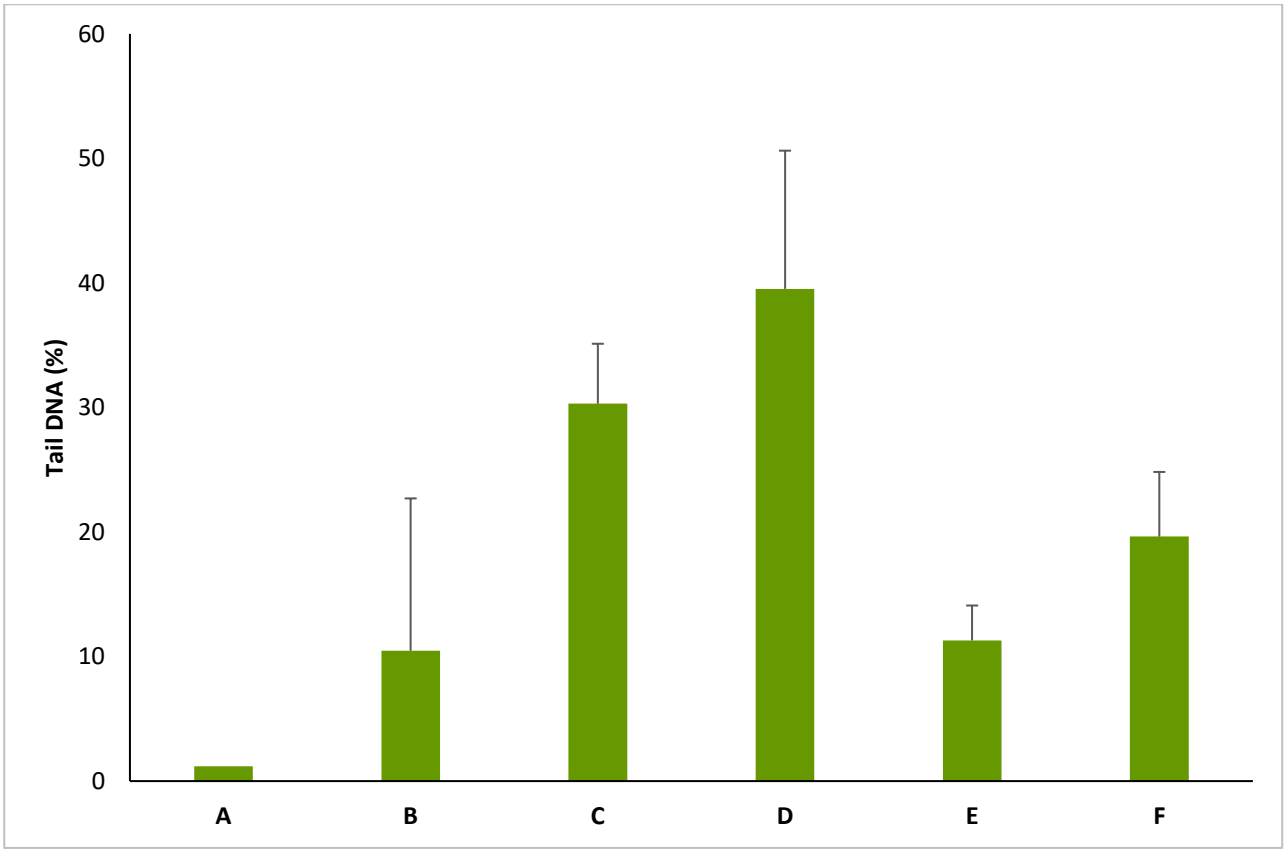


**Figure 3.1.** Comet assay results obtained on fresh blood with different laboratory conditions. N=3 for each experimental condition.

The figure 3.1 shows the results obtained for the comet assay with the different parameters modified. Regarding the dilutions, all the conditions showed a highest DNA damage with two dilutions with values greater than 30%, which is not acceptable as a baseline value, since internationally recognized values for the control of comet assay must be below 10-12% of DNA damage. Only the use of the condition "E" (1:1000 and 1:4) showed 10% of DNA damage. The results of the electrophoretic run times at 15 minutes showed values too high compared to the same

conditions shown with 10 minutes of running. Considering all the results as a whole, the following parameters were defined: two dilutions (1:1000 and 1:4) and 10 minutes of electrophoresis time.

*Comet assay with frozen blood of Common Krestel*

Once the test has been developed on fresh blood, the first step to develop the method for comet assay on frozen blood samples was to determine the proper dilution in a cryopreservation medium (DMSO:RPMI, 20:80). The cryopreservation mixture was already developed and used on other species and it gave excellent results. Usually, the blood is diluted in the cryostorage mixture to preserve the viability of the cells and prevent the formation of ice crystals that could form during the freezing of the blood itself. The dilution rate may, however, vary from species to species; in this case, the comet assay was carried out on samples stored with the following concentrations between blood and cryoconservation mixture: 1:2, 1:5, 1:10, 1:20, 1:50, 1:100 and 1:200. The concentration 1:200 tested was the best while with the other samples were illegible (Figure 3.2).

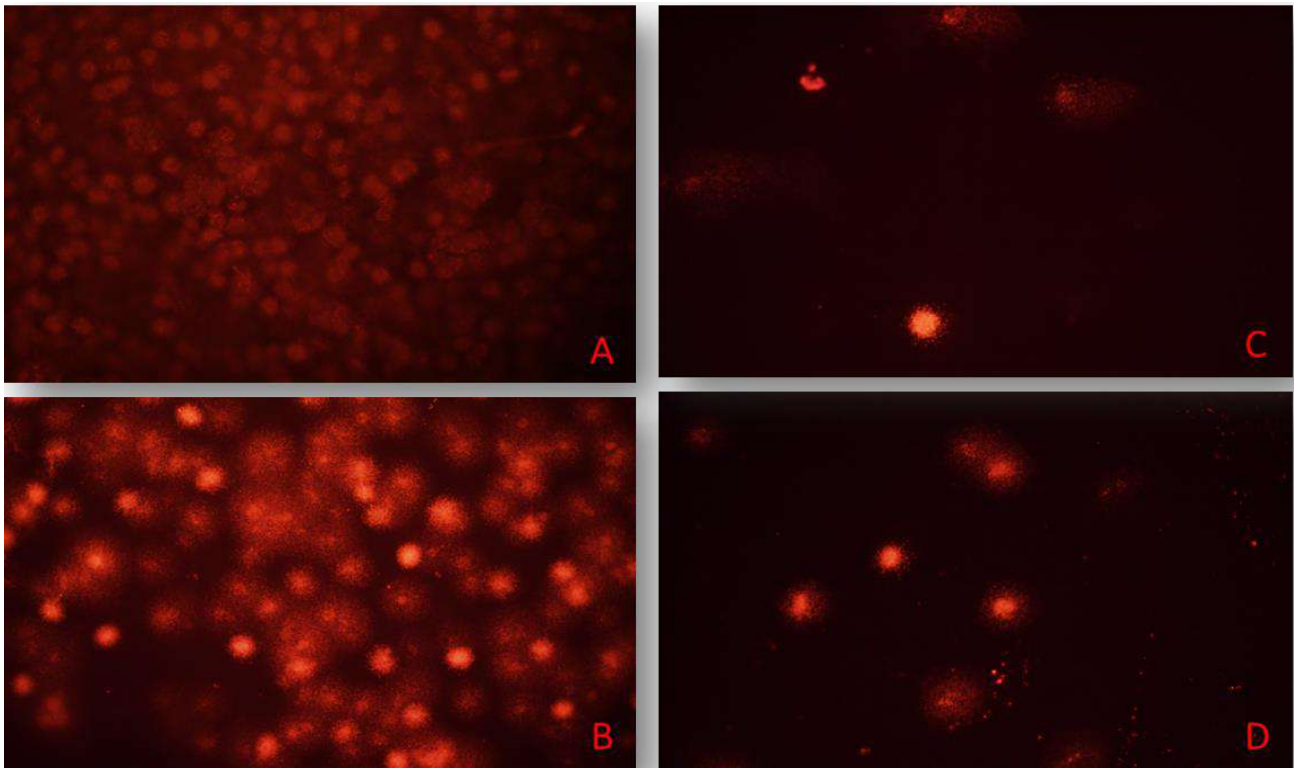


**Figure 3.2.** Comet assay applied on frozen blood samples differently diluted with cryopreservation mixture. A, B: dilutions 1:2, 1:5, 1:10, 1:20, 1:50, 1:100; C,D: 1:200 dilution.

Once the conditions of cryoconservation of the blood were established, the parameters developed for the fresh comet assay were tested and confirmed.

*Respiratory burst*

The respiratory burst process in Common Kestrel individuals was evaluated for the first time in this research study. The assay involves the use of a salt test, NBT (nitro blue tetrazolium), which in the presence of ROS is reduced to formazan blue that precipitates into crystals within the cells that accomplish respiratory burst, thus allowing the measurement of the intracellular ROS produced. The method is semi-quantitative and is expressed as absorbance at 630 nm read in the solution resulting from the dissolution of the intracellular formazan blue. This assay was developed in Kestrel by modifying the method of Caliani et al. (2019) applied to *Caretta caretta*.

Three modifications were made to the method: the incubation temperature of the plate, the stimulation of the cell membranes of leukocytes performing respiratory burst with PMA (Phorbol 12-myristate 13-acetate), and the amount of blood sample. For the first aspect, we keep a temperature of birds; regarding the membrane activator, we chose to use it in Common Kestrels samples because the superoxide anion, in the case of birds, is also generated by the mitochondrial activity of red blood cells thus we needed to stimulate the lymphocytes membranes. Finally, we tested the different amounts of samples (100, 50, 30, 20, and 10 µL) and no significant differences emerged; thus, we chose 20 µL so that the remaining blood could also be used for other assays.

### 3.4. *In vitro* laboratory studies

The main purpose of *in vitro* laboratory studies was to assess the potential genotoxicity of certain classes of pesticides on birds by applying the comet assay, a genotoxicity biomarker. To achieve this objective, laboratory studies were conducted on blood samples from individuals of Common Kestrel housed in a LIPU centre and Raptors rescue centre. Blood (150 µL) was sampled from the jugular vein by a veterinarian using a 1 mL syringe and transferred in a vacutainer (MICROMED®, Italy) containing lithium-heparin as anticoagulant. The compounds tested in this experiment, as commercial formulations, were a fungicide (Amistar®Xtra) and an herbicide (Round up). Ethyl methane-sulfonate (EMS), a well-known genotoxic compound was used as positive control.

Graphs have been elaborated with Microsoft excel. The data obtained by the laboratory studies were processed by statistical analysis using the Kruskal-Wallis and the $Chi^2$ tests to highlight the differences between treatened group and control. Statistical analysis were conducted through the free software Past4.03.

The first step of the experiment was to test the viability of blood cells exposed to the two pesticides. For the experiment with Amistar®Xtra, the concentration tested was 200 g/L, which is the

concentration used in the field. Based on the present bibliography, the blood was exposed to this compound for 1, 2, 3, 5, 6 and 24 hours. The figure 3.3 shows that control cells maintained 100% viability at all times of exposure. The positive control vitality was 100% up to six hours, while it collapsed to 0% at 24 hours of exposure. Concerning the cells exposed to the fungicide, at T0 a vitality of 100% was observed, value that remained constant until 6 hours of exposure and then was reduced to 60% at 24 h of exposure. It was then determined that an exposure of up to 6 hours may be sufficient to observe DNA effects on cells.


**Figure 3.3.** Cell viability (%) of blood cells of Common Krestel exposed to the fungicide, Amistar (200 g/L) for 1, 2, 3, 5, 6 and 24 hours. The line orange indicates the control, the yellow the positive control (EMS) and the green the Amistar at 200 g/L of concentration.

Concerning the glyphosate, the dose used in the field was tested (300 mL/L). The blood cells were exposed to the herbicide for 1, 2, 3 and 24 hours. The control showed 100% viability at all exposure times, while the positive control showed a decrease in viability after 3 hours. After an hour of exposure glyphosate showed an 80% viability, after 3 hours at 40% and at 24 hours all blood cells were dead (Figure 3.4). It was then decided to decrease the concentration of glyphosate to see if cell viability was maintained for more than an hour.

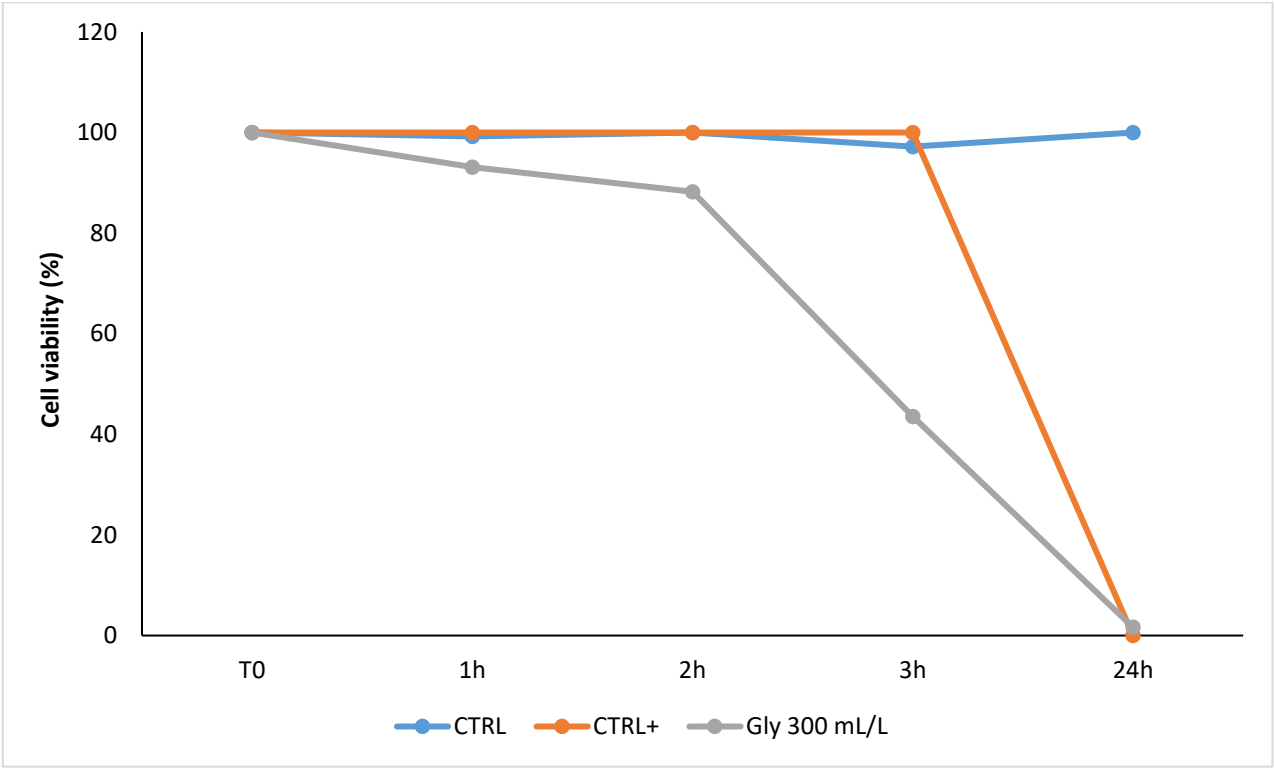


**Figure 3.4.** Cell viability (%) of blood cells of Common Krestel exposed to the erbicide, glyphosate (300 mL/L) for 1, 2, 3 and 24 hours. The line blue indicates the control, the orange the positive control (EMS) and the grey the glyphosate at 300 mL/L of concentration.

Concentrations below 300ml/L glyphosate were chosen: 75 mL/L, 18.75 mL/L and 4.68 mL/L. As in previous experiments, control and positive control showed 100% cellular viability. Treatments with 75 mL/L and 18.75 mL/L showed very low vitality already after 10 minutes, while the dose at 4.68 ml/l showed excellent vitality for all exposure times (Figure 3.5). The lowest dose was chosen and tested for comet assay.

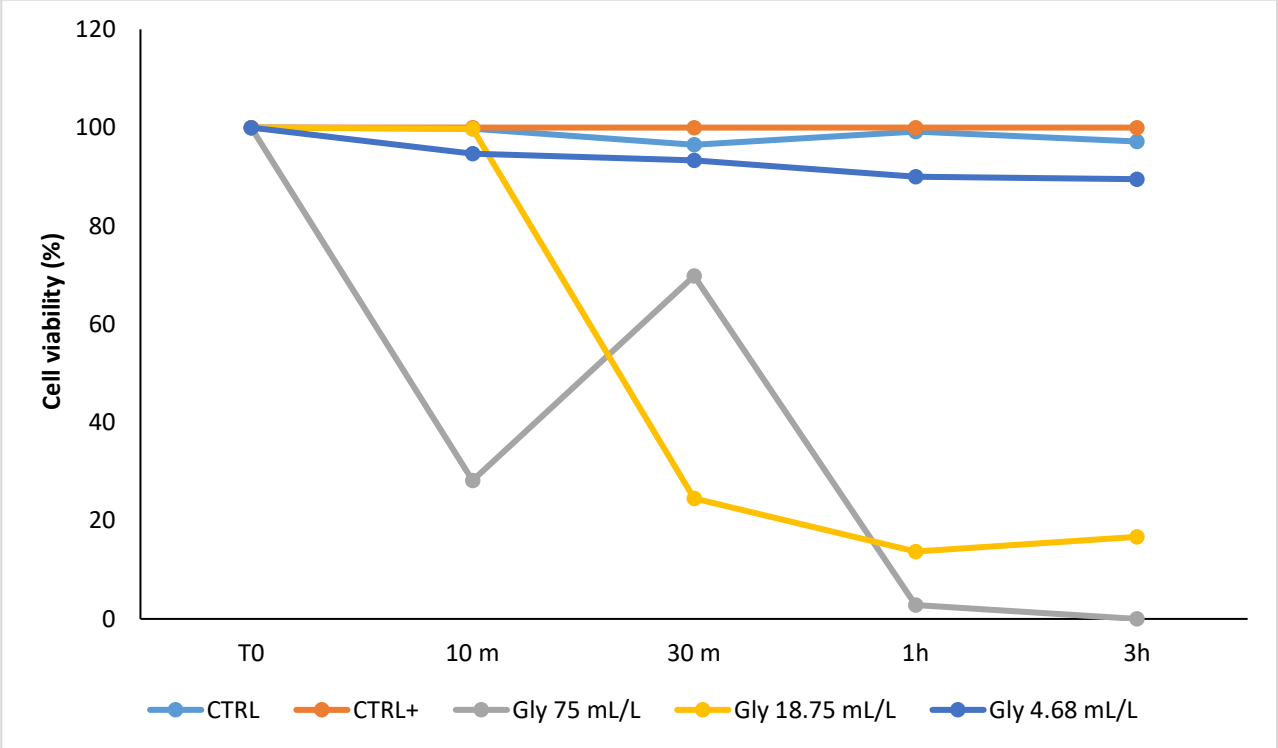


**Figure 3.5.** Cell viability (%) of blood cells of Common Krestel exposed to the erbicide, glyphosate at different concentrations (75 mL/L; 18.75 mL/L; 4.68 mL/L) for 10 min, 30 min, 1 h and 3 hours. The light blue line indicates the control, the orange the positive control (EMS), the grey the glyphosate at 75 mL/L of concentration, the yellow the glyphosate at 18.75 mL/L and the blue the glyphosate at 4.68 mL/L of concentration.

Figure 3.6 shows the results after exposure of Common Krestel blood cells at a dose of 200 g/L Amistar®Xtra for 1, 3 and 6 hours. At all exposure times, DNA damage values for the fungicide are higher than the respective controls; a statically significant difference was found between control and Amistar®Xtra at 1 hour of exposure ($p<0.05$).

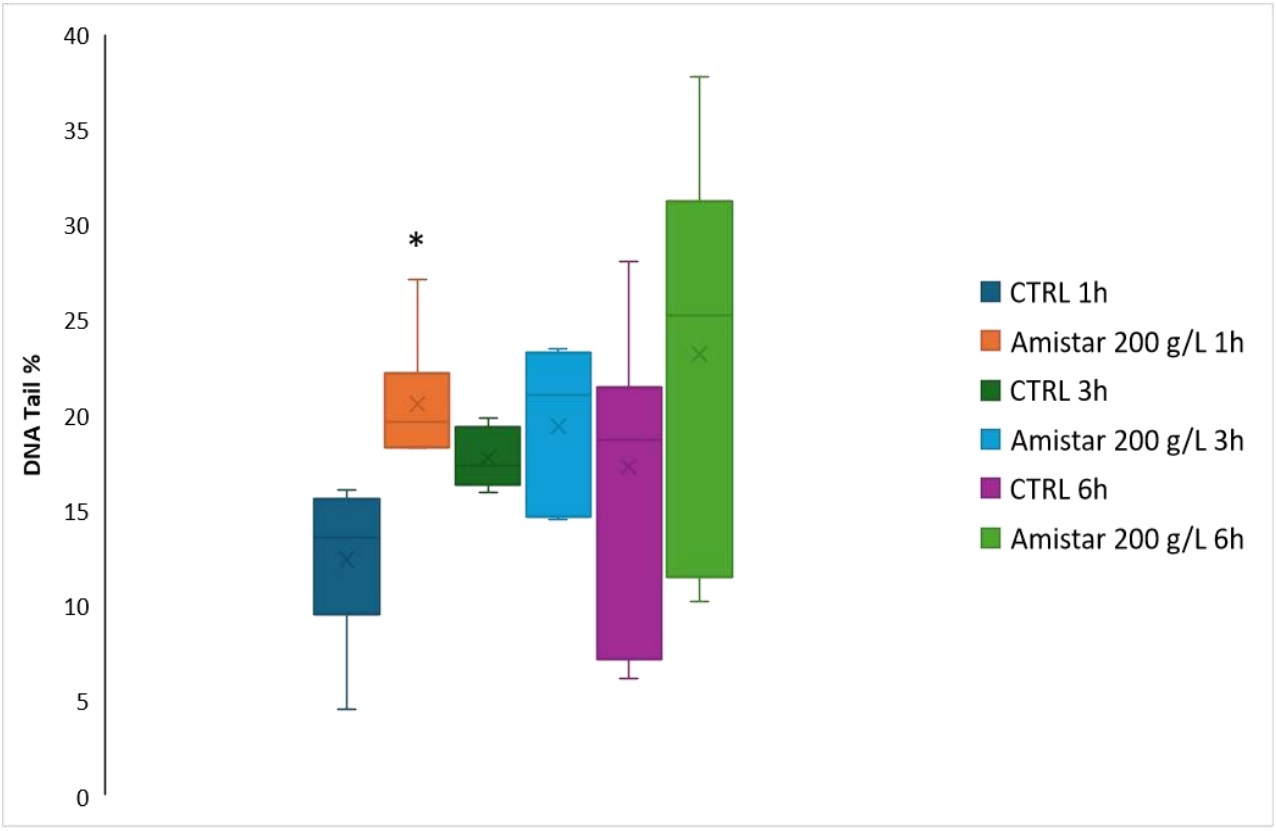


**Figure 3.6.** DNA tail % measured in blood of Common Krestel exposed to Amistar®Xtra (200 g/L) for 1, 3 and 6 hours. * indicates a statistically significant difference of exposed cell for 1 hour respect to control ($p<0.05$).

In the glyphosate experiment, at both exposure times the control values are lower than those of the respective treatments and as the exposure time increases, the genetic damage values increase. At 3 hours a statistically significant difference was found between control and exposed cells (Figure 3.7).

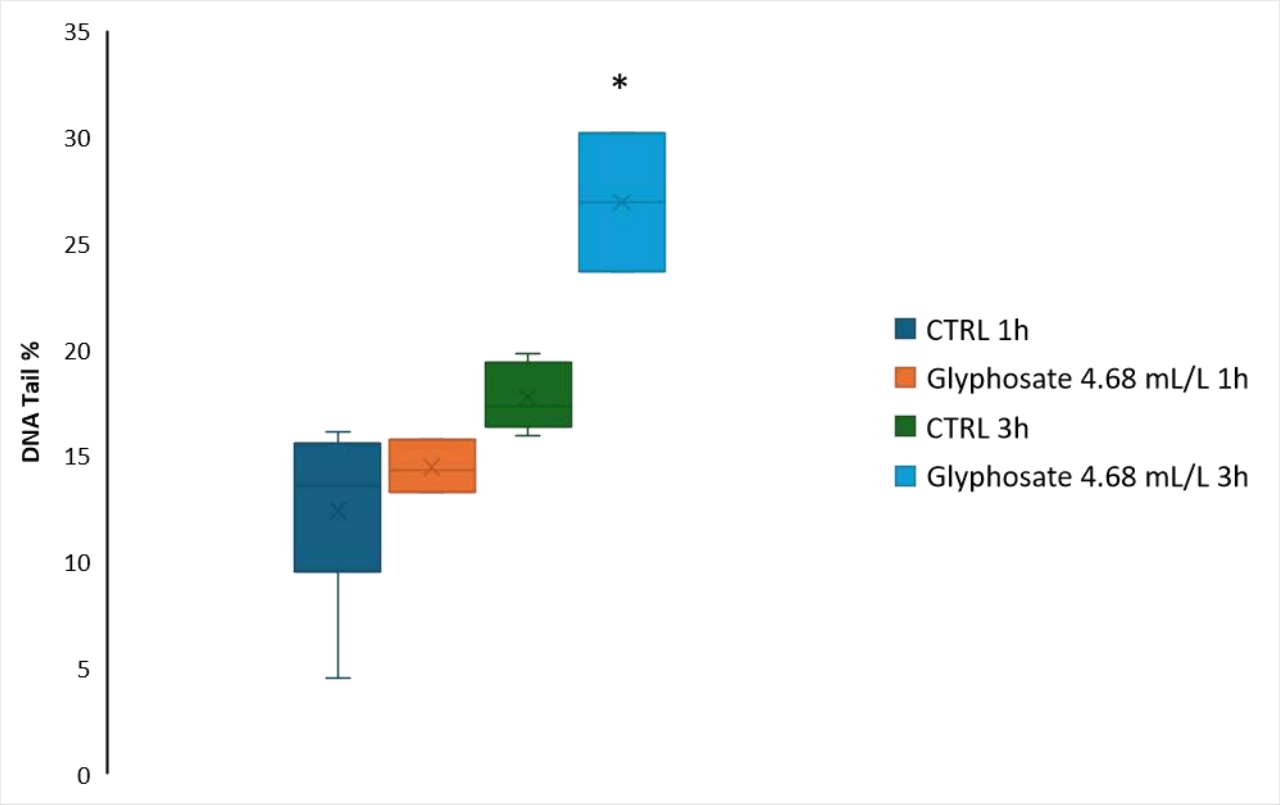


**Figure 3.7.** DNA tail % measured in blood of Common Krestel exposed to glyphosate (4.68 mL/L) for 1 and 3 hours.
* indicates a statistically significant difference of exposed cell for 3 hours respect to control ($p<0.05$).

This experiment allowed to validate the Comet Assay in this species. Marked differences were found in the cell viability and in the genotoxic potential, after exposure to the two different compounds (using usage doses). The fungicide resulted much less toxic in comparison to glyphosate.

Glyphosate was found to be strongly toxic to cells even at concentrations well below usage doses and for very short times of exposure. Being glyphosate the widest used herbicide, with controversial opinions about it toxicity potential, it is worth to underline that this first step of *in vitro* studies confirm the need to deepen *in vitro* and *in vivo* studies on the toxicity of this compound to non target species.

## 3.5. A blood-based multi-biomarker approach reveals different physiological responses of common kestrels to contrasting environments

This paragraph concerns the monitoring study conducted on Common Kestrels populations from different areas of the Lazio Region, characterized by different anthropic pressures. The status of the kestrel populations was assessed using a blood-based multi-biomarker approach. This study is reported in the present paragraph as the pdf of the paper publication entitled “A blood-based multi-biomarker approach reveals different physiological responses of common kestrels to contrasting environments”, published on Environmental Research by Giovanetti et al., 2024. The supplementary material of this paper is also included in the present thesis as reported in the online version of the article (Section “supplementary material”).

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# A blood-based multi-biomarker approach reveals different physiological responses of common kestrels to contrasting environments☆

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ABSTRACT

The increase of urbanization and agricultural activities is causing a dramatic reduction of natural environments. As a consequence, animals need to physiologically adjust to these novel environments, in order to exploit them for foraging and breeding. The aim of this work was to compare the physiological status among nestling common kestrels (*Falco tinnunculus)* that were raised in nest-boxes located in more natural, rural, or urban areas in a landscape with a mosaic of land uses around Rome in Central Italy. A blood-based multi-biomarker approach was applied to evaluate physiological responses at multiple levels, including antioxidant concentrations, immunological functions, genotoxicity, and neurotoxicity. We found lower concentrations of glutathione and GSH:GSSG ratio values and higher proportions of monocytes in urban birds compared to the other areas. We also found higher DNA damage in rural compared to urban and natural krestels and inhibition of butyrylcholinesterase activity in urban and natural birds compared to rural area. Finally, we found similar values among study areas for respiratory burst, complement system, bactericidal capacity, and plasma non-enzymatic antioxidant capacity. These results suggest that (i) city life does not necessarily cause physiological alterations in kestrels compared to life in other habitats, and (ii) environmental pressures are likely to differ in typology and intensity across habitats requiring specific responses that a multi-biomarker approach can help to detect. Further studies are needed to assess which factors are responsible for the physiological differences among city, rural, and natural birds, and whether these differences are consistent across time and space.

## 1. Introduction

Increasing world population with consequent expansion of urban and agricultural areas has caused profound changes to the natural environments raising novel challenges for wildlife (Isaksson, 2020; Moreau et al., 2022). City life exposes wildlife to several potential stressors that are less common in other environments, such as chemical and noise pollution, artificial light at night, and human presence. In addition, other factors can differ between urban and natural environments, such as food quality and availability, exposure to pathogens, and predation (Isaksson, 2018). As a consequence, animals need to adjust their physiology and behaviour in order to adapt to the novel environmental conditions that they encounter in the city, determining a differentiation between conspecific urban and rural populations. For example, early experimental studies carried out under common garden conditions found that urban blackbirds (*Turdus merula*) show a reduced stress responsiveness (Partecke et al., 2006) and lower levels of blood oxidative damage (Costantini et al., 2014a) compared to rural blackbirds. Studies on free-living birds also identified important differences between urban and non-urban populations. Studies on common kestrels (*Falco tinnunculus*) found that urban birds had altered body colourations (Sumasgutner et al., 2018) and were in lower body condition (Wemer

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et al., 2021) compared to rural birds.

Living outside a city is not cost-free. Agricultural activities can also affect wildlife owing to natural habitat loss, harvesting operations, disturbance, pesticides use and toxicity, and reduction of food supply (reviewed by Stanton et al., 2018). Variations in food availability and quality can trigger cascading events that reduce the individual physical wellness and breeding success. For example, landscape homogenization due to agricultural intensification can disrupt the relationship between vole (*Microtus agrestis*, *Microtus rossiaemeridionalis* and *Myodes glareolus*) abundance and reproductive success in birds of prey (Sumasgutner et al., 2019). Habitat change can alter the feeding behaviours of several avian species, impairing reproductive success (Sumasgutner et al., 2019; Sinkovics et al., 2021; Solís et al., 2023) and altering the body condition and physiological status of nestlings (Costantini et al., 2014b; Almasi et al., 2015; Isaksson et al., 2018; Roos et al., 2021).

These studies show that bird species can widely differ in their resilience or adaptability to city life. The question then is which factors lead individual species to flourish or suffer in urban habitats. The common kestrel (thereafter "Kestrel") is a diurnal raptor distributed in open lands (e.g., grassland and farmland) and urban environments of Eurasia and Africa (Costantini and Dell'Omo, 2020). It is a predator with a varied and variable diet depending on the geographic region and prey availability. It feeds manly on small mammals in northern Europe (e.g. voles); by contrast, it shows a wide trophic niche in southern regions (Costantini and Dell'Omo, 2020). Despite its classification as "Least Concern", some European populations show a modest but constant decline since the 1980s (BirdLife International, 2021). It has been suggested that the urban habitat is not optimal for kestrels, probably because of a scarcity of preferred prey or chemical contamination (Grande et al., 2018; Kettel et al., 2018). However, kestrels can also face with other important threats outside the city, such as, landscape simplification and homogenization due to agricultural intensification (Costantini et al., 2014b; Butet et al., 2022), burden of ectoparasites (Wemer et al., 2021), and pesticide exposure (Roos et al., 2021). The Kestrel, being a top predator, can accumulate higher amounts of persistent organic contaminants and undergo processes of biomagnification compared to species at the lowest levels of the food web (Yu et al., 2013; Rial-Berriel et al., 2020, 2021; Rodríguez Álvarez et al., 2022; Zhang et al., 2021). Thus, kestrels can be exposed to multiple and diverse stressors across urban, rural, or natural habitats, so that it is not straightforward to define general predictions or conclusions about which habitat would be less challenging.

Since multiple stressors could affect kestrels, it is important to rely on multiple methods that enable the investigation of how diverse environmental changes, induced by human activities, can influence the physiology of birds. Biomarkers are recognized sensitive tools for measuring biological effects, assessing environmental quality, and revealing the overall toxicities of complex mixtures (Martinez-Haro et al., 2015; Milinkovitch et al., 2019). Considering the key role of physiological systems that enable individuals to cope with new selective pressures, such as those encountered in urban areas, it has been suggested that physiological biomarkers may contribute to detect early warning signals of possible long-term effects on the population (Fossi and Leonzio, 1994) and to evaluate the sensitivity or resilience of wildlife to urbanisation (Isaksson, 2020).

Relying on a blood-based multi-marker approach, we measured the physiological responses of kestrels to the city life at multiple levels, including immunological function (respiratory burst, differential white blood cells (WBCs) count, heterophils and lymphocytes (H/L) ratio, complement system activity, bacterial killing assay), oxidative status (total glutathione tGSH, reduced and oxidized glutathione GSH and GSSG, the ratio GSH:GSSG, the non-enzymatic antioxidant capacity and the Total Antioxidant Status (TAS)), genotoxicity (comet and erythrocytes nuclear abnormalities (ENA) assays) and neurotoxicity (butyrylcholinesterase and carboxylesterase (BChE and CaE) activities). We relied on these particular biomarkers because previous work showed that they can vary in relation to environmental quality or individual health (Isaksson et al., 2005; Casini et al., 2006; Bonisoli-Alquati et al., 2010; Oudi et al., 2019; Nwaogu et al., 2023) To this end, we compared these multiple biomarkers among kestrel populations living in more urban, rural, or natural habitats. In so doing, we could also assess whether kestrels face with environment-specific challenges and determine the severity of such challenges.

## 2. Material and methods

### 2.1. Study area and urban gradient calculation

We carried out this study in three areas in and around Rome characterized by a mosaic of land uses and human exploitation. These areas were classified as natural site (Castel di Guido, NAT), rural site (Decima Malafede, RUR), and urban site (Aniene Park, URB) (Fig. 1), relying on the quantification of the urban gradient, calculated as a percentage of sealed surfaces (buildings and roads) following Sumasgutner et al. (2014) and Kübler et al. (2005). Specifically, we calculated the urban gradient by applying the land-use Corine Land Cover classes with buffer zones of 500 m radius around nests (QGIS v3.10 software). The value of a 500 m radius was chosen based on GPS-tracking data reported by Damiani et al. (2022) on the home range of kestrels in our study area.

Castel di Guido (1.98 % of sealed surface) is located within the *Litorale Romano* Natural Reserve, at 20 km from Rome city center. The area is dominated by meadows, natural grasslands with free-ranging sheep, wooded zones (*Quercus* sp.), Mediterranean scrub and, to a smaller degree, wheat crops (Blasi, 1994). Decima Malafede (5.25 % of sealed surface) is a Natural reserve at about 10 km from Rome. It is located among several busy roads (Grande Raccordo Anulare, Pontina, and Laurentina roads) and is characterised by wheat crops and grasslands used by free-ranging sheep. The Aniene Park (44.49 % of sealed surface) is entirely within Rome city and is crossed by the homonymous river. The river flows into some of the most densely populated districts of the city (Dell'Omo et al., 2008). Thus, the three sites are located in a landscape with a mosaic of land uses, so that none can be considered fully representative of a natural or rural habitat. However, given the different land uses of the three locations, we can consider one site to be more natural or urban than another site.

### 2.2. Sample collection

We collected blood samples from a total of 64 nestlings, belonging to 19 nests, in May and June of 2020 and 2021. Specifically, we sampled a total of 30 nestlings from the NAT area (10 nests), 26 from the RUR area (7 nests) and 8 from URB area (2 nests). Nestlings were raised into nestboxes installed on power lines (Terna s.p.a.) and monitored by *Ornis italica* since 2000s.

We collected the blood (500 μL) from the metatarsal vein using 1 mL syringes and immediately transferred it into vacutainers (MICROMED®, Italy) containing lithium heparin as anticoagulant. We used a drop of fresh blood to make blood smears (two per bird). Straightaway, we froze an aliquot of whole blood, and we mixed a second aliquot, destinated for the comet assay, with a cryoconservation medium (20:80 DMSO:RPMI) to prevent cells from mechanical damage and to keep the cells viable. The remaining whole blood was centrifuged at 2000 *g* (Multispin 12, Argo LAB) to separate plasma from red cells. We stored all the samples at −80 °C until analyses.

### 2.3. Laboratory analyses

#### 2.3.1. tGSH and GSSG

The tGSH and its oxidized form were measured in whole blood following Isaksson et al. (2005). 4 μL of sample were mixed with 16 μL of 5% sulfosalicylic acid (SSA) and centrifuged at 10000 rpm for 10 min at 4 °C. 10 μL of the supernatant were further diluted (1:20) with

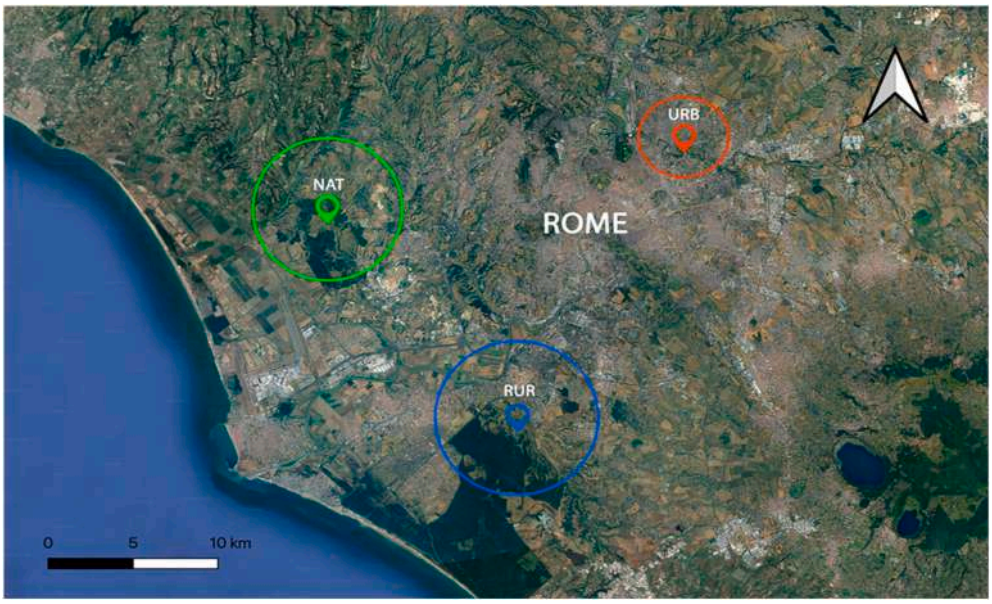


**Fig. 1.** Study areas of common kestrel nestlings located in the Lazio Region: NAT (green), RUR (blue) and URB (red).

GSH-buffer (143 mM $NaH_2PO_4$, 6.3 mM EDTA, pH 7.4). For GSSG content, 100 μL of the above dilution were mixed with 5 μL of 4-vinylpyridine (4-VNP), incubated for an hour at room temperature and centrifuged at 12000 rpm for 5 min at 4 °C. GSH standard curve was prepared by diluting the GSH stock solution (10 mM) with 0.5% SSA to the following concentrations 1.875, 3.12, 6.25, 12.5, 25, 50 and 100 μM. GSSG standards were prepared by diluting a 5 mM stock solution to the concentrations 0.1875, 0.3125, 0.625, 1.25, 2.5, 5 and 10 μM. All samples and standard curves were run in duplicate using 96-well plates. 20 μL of sample or standard, 200 μL of the working solution (10 mM DTNB, 172 and 174.5 μL of GSH-buffer for tGSH and GSSG, respectively and 0.34 and 0.17 units/well of GR for tGSH and GSSG, respectively) and 34 μL of 2 mM NADPH were added to each well. Thereafter, the plate was placed in a multiplate reader (Multiskan Skyhigh Thermo Scientific), and absorbance measured at 412 nm at room temperature every 30 s for 5 min and compared to a simultaneously obtained standard curve from GSH and GSSG with known concentration. We calculated the concentration of GSH (i.e. reduced glutathione) by subtracting GSSG values from tGSH values. Results were expressed as μM.

### 2.3.2. TAS assay

The TAS was evaluated by the use of a commercial kit (Antioxidant Assay Kit, Sigma, St. Louis, MO) based on the method of Miller et al. (1993) and modified by Caliani et al. (2019). A stock solution of 1.5 mM of Trolox, a water-soluble analogue of vitamin E, was used for the standard curve. Trolox was diluted in assay buffer for the preparation of the different standard curve points (0, 0.015, 0.045, 0.105, 0.21, 0.42 mM). Aliquots of each concentration (10 μL/well) were added to a 96-well plate in duplicate. For each sample 10 μL of plasma diluted 1:100 in assay buffer were added in duplicate to the plate. 20 μL of myoglobin and 150 μL of chromogen [ABTS (2,2-Azino-di [3-ethylbenzthiazoline])] were added to each well and the plate incubated at room temperature for 4.5 min. Absorbance at 405 nm was measured using a multiplate reader (Multiskan Skyhigh Thermo Scientific). The TAS was expressed as mM of Trolox by linear regression of the standard curve.

### 2.3.3. Respiratory burst

The respiratory burst activity was evaluated as the presence of intracellular oxyradical produced by NADPH oxidase, and it was measured with the Nitroblue Tetrazolium (NBT) assay, following the method of Caliani et al. (2019), modified. For each sample, 20 μL of whole blood were added in duplicate to a 96-well plate and incubated at 40 °C for 2 h to allow cell adhesion. Unattached cells were then washed off 3 times with L-15 medium. 100 μL of L-15 medium supplemented with NBT (1 mg/mL) and phorbol 12-myristate 13-acetate (PMA; 100 μg/mL) was then added to each well and the plate was incubated at room temperature for 1 h. After incubation, the plate was discarded and fixed with 100% methanol for 10 min. The plate was washed several times with 70% methanol and air-dried. 120 μL of KOH and then 140 μL of DMSO were added to each well in order to destroy cell wall and dissolve the crystals of formazan blue deriving from the reduction of NBT by the oxyradicals. Measurements of absorbance were performed at 630 nm using a multiplate reader (Multiskan Skyhigh Thermo Scientific) and KOH/DMSO as blank (120 μL of KOH and 140 μL of DMSO). The respiratory burst activity was expressed as a reduction of NBT (optical density at 630 nm).

### 2.3.4. Differential white blood cells (WBC) count

Air-dried blood smears were stained with Diff-Quick stain (Mgg Quick Stain, Bio-optica), air-dried and two hundred leukocytes were manually counted using an optical immersion microscope (Olympus BX41) at 100x magnification. Leukocytes were classified as heterophils, eosinophils, basophils, lymphocytes and monocytes according to the cellular morphology for birds described by Campbell (1995).

### 2.3.5. H/L ratio

The ratio between the number of heterophils and lymphocytes was measured for each animal, in order to obtain H/L ratio, a physiological stress index.

### 2.3.6. Complement system activity

Plasma complement activity was determined by the method of sheep red blood cells (SRBCs) hemolysis following the protocol of Merchant and Britton (2006). Whole blood collected from a healthy Merino sheep (*Ovis aries*) at a private sheep farm was centrifuged at 3000×*g* to obtain fresh SRBCs. The SRBCs were washed with phosphate-buffered saline (PBS, pH 7.4) several times until supernatant was clear, then diluted to 2% (v/v) in PBS. Common kestrel plasma was then incubated with an equal volume of 2% SRBC (v/v) for 30 min at 37 °C. Thereafter, the sample was centrifuged at 2500 g for 5 min. 40 μL of supernatant were used in microplate reader at 540 nm (Multiskan Skyhigh Thermo

Scientific). As a positive control, 2 μL Triton X-100 was added to 700 μL of a 1% SRBC suspension and homogenized until complete hemolysis; then, it was centrifuged and the optic density (O.D.) measured (considered 100% hemolysis). The results were expressed as mean % hemolysis=(O.D. sample/O.D. positive control) × 100.

#### 2.3.7. Bacterial killing assay

We measured the plasma bactericidal capacity following the French et al. (2010) method with some modification. 10 μL of plasma were diluted (1:2) in sterile phosphate-buffered saline (PBS). The *E. coli* stock solution ($10^6$ bacteria/mL) was prepared by dissolving a single lyophilized pellet (ATCC 8739; 0483E7; Epower Microorganisms, Micro-iBioLogics) in 10 mL of sterile PBS. This was further diluted in PBS to $10^5$ bacteria/mL working solution on day of analysis. Each plasma replicate was mixed with 5 μL of *E. coli* working solution. Positive controls consisted of 5 μL of working solution with 10 μL of PBS, whereas negative controls only 15 μL of PBS. Samples and controls in duplicate were incubated at 37 °C overnight for 12 h in 96-well round-bottom plates. Absorbance was measured at 300 nm using a microplate reader (Multiskan Skyhigh Thermo Scientific). We calculated the percentage of bacteria killed as follows: 1 - (sample mean/positive control mean) x 100.

#### 2.3.8. Comet assay

Erythrocytes were processed for the comet assay according to Caliani et al. (2014) with some modifications. Blood was diluted in PBS (1:4000), embedded within agarose gel (0.5% low-melting agarose) and layered on two slides per sample, pre-dipped in 1% normal melting agarose. The slides were immersed into a freshly made lysis solution (2.5 M NaCl, 10 mM Tris, 0.1 M EDTA, 1% Triton X-100, and 10% DMSO, pH 10) for at least 1 h at 4 °C in the dark. The slides were then placed on a horizontal electrophoresis tray previously filled with freshly prepared cold alkaline buffer and left for 15 min to allow DNA unwinding. Electrophoresis was performed at 16 V and 250 mA for 10 min. Slides were then neutralized (3 × 5 min; 0.4 M Tris, pH 7.5) and stained with SYBR®Safe 1:10.000 in TE (10 mM Tris-HCl pH 7.5 and 500 mM EDTA pH 7.5) buffer. To prevent DNA damage by photolysis, all phases were conducted under yellow light or in the dark. A total of 100 cells per sample were examined under the epifluorescence microscope (Olympus BX41) at 40 × magnification. The amount of DNA damage was evaluated as the DNA tail % using an image analyzer (6.0 Software, Kinetic Imaging Ltd.).

#### 2.3.9. ENA assay

The ENAs assay allows to assess the presence of chromosomal aberrations generated during mitosis by the chromosome breakage or failure cytokinesis in erythrocytes (Herek et al., 2021). Aberrations, such as micronuclei, indicate consolidated damages (Caliani et al., 2009). Quantification of ENAs was conducted in the same slides used for differential leukocytes count according to the method of Frixione and Rodríguez-Estrella (2020). A total of 2000 mature erythrocytes per sample was analysed and manually quantified at 100x magnification using an optical immersion microscope (Olympus BX41). We classified the nuclear alterations as follow: bud, segmented, notched, kidney and micronucleus (MN). Results were expressed as a total number of abnormalities per 2000 cells.

*2.3.9.1. BChE activity.* Butyrylcholinesterase (BChE) is an enzyme inhibited by organophosphates (OPs) and carbamates (CBs) and provide early responses to the exposure of neurotoxic compounds (Anglister et al., 2023). It is a sensitive biomarker of indirect effect of neurotoxic compounds because its inhibition can be indirectly related to AChE (acetylcholinesterase) inhibition (Fossi et al., 1992). BChE activity was evaluated in plasma samples using the spectrophotometric test of Fossi et al. (1992). The reaction assay mixture contained: 25 mM TRIS-HCl buffer (pH 7.6), 1 mM $CaCl_2$; DTNB (5, 5′dithiobis 1-2-nitrobenzoic acid), 10 μL of BTCI (butyrilthiocoline iodide) and 2 μL of plasma. Samples were run in double using a 96-well plate. Absorbance was measured at 410 nm with a microplate reader (Multiskan Skyhigh Thermo Scientific) at 40 °C every 30 s for 5 min. The activity was expressed as μmol/min/mL plasma.

#### 2.3.10. CaE activity

Carboxylesterases (CaEs) are enzymes that are able to offer alternative binding sites for insecticides to prevent AChE inhibition and participate to phase I metabolism by converting substances into more soluble forms for easier excretion (Casey Laizure et al., 2013). CaE activity was evaluated by the method of Solé et al. (2022), with minor modifications. To 96-wells plate we added the reaction assay mixture, which contained: phosphate buffer 100 mM (pH 7.4), the substrate p-nitrophenyl butyrate (pNPB) 1 mM and 5 μL of plasma. Samples and blank were run in double. A microplate reader (Multiskan Skyhigh Thermo Scientific) was used for measuring absorbance at 405 nm at 40 °C every 30 s for 5 min. The activity was expressed as nmol/min/mL plasma.

### 2.4. Statistical analyses

We used linear mixed models fit by REML using the package lme4 (Bates et al., 2015) in R Studio (R Core Team, 2021) to test the variation of markers among the three sites. In each model, we included the area as fixed factor. To control for any confounding effects of other factors, we also included the year of blood collection as fixed factor, and brood size and laying date as covariates. Both covariates were centered within each sampling year. We also included the nest as random factor. We used the package emmeans to calculate marginal means and standard error of each marker by sampling area. All models respected the assumptions of normal distribution of residuals and lack of collinearity. We used the compute.es package (Del Re, 2013) in R version 4.0.5 to calculate the standardized effect size Hedges' g and credible intervals for comparisons between sites. Effect sizes were considered small when g = 0.2, intermediate when g = 0.5, or large when g = 0.8 (Cohen, 1988; Møller and Jennions, 2002). Of each biomarker, we also reported mean, standard deviation, and range in the supplementary table (Table S1).

## 3. Results

As shown in Table 1 and Fig. 2, the GSH levels and GSH:GSSG values were significantly lower in nestlings from URB than those from NAT (GSH = $p = 0.00073$; GSH:GSSG = $p = 0.038$) and RUR (GSH = $p < 0.001$; GSH:GSSG = $p = 0.038$), whereas GSSG and TAS did not show significant differences among areas (Fig. S1). Leukocytes classes and H/L ratio were not significantly different among areas (Table 2, Fig. S2), with the exception of monocytes. Nestlings from URB showed higher values of monocytes ($p = 0.032$) than nestlings from NAT but no significant differences emerged with RUR (Table 2, Fig. 3). The other biomarkers of immune system did not significantly differ among areas (Table 2, Fig. S2), although complement system activity tended to be lower in URB than in RUR ($p = 0.097$). Regarding the genotoxicity biomarkers, the values of comet assay were significantly higher in RUR than NAT ($p = 0.012$) and URB ($p = 0.008$) (Table 3, Fig. 4). A similar trend was observed for the total ENA assay, but without significant differences among areas (Table 3, Fig. S3). Finally, BChE activity values were significantly lower in nestlings from URB ($p = 0.006$) and NAT ($p = 0.045$) than those from RUR (Table 4, Fig. 5). CaE activity did not show any significant differences among study areas (Table 4, Fig. S4). Estimates of effect sizes for the significant differences in biomarker values between sites were ≥0.8, indicating that these differences were large (Table 5). Moreover, effect sizes were significant because their credible intervals did not overlap zero.

All oxidative status markers were significantly higher in 2020 than

**Table 1**
Outcomes of linear mixed models used to examine the effects of breeding areas differing in land cover and anthropogenic pressures on oxidative status markers in common kestrel (*Falco tinnunculus*) nestlings. *$P < 0.05$, significant difference. NAT = natural, RUR = rural, URB = urban.

| Variable | Factor | Reference Level | Level | Coefficient Estimate ±SE | *t* | *p* |
|---|---|---|---|---|---|---|
| GSH | | | | | | |
| | Area | NAT | RUR | 0.248 ± 0.235 | 1.055 | 0.299 |
| | | NAT | URB | −1.083 ± 0.291 | −3.713 | <0.001* |
| | | RUR | URB | −1.331 ± 0.234 | −5.678 | <0.001* |
| | Sampling year | 2020 | 2021 | −1.290 ± 0.177 | −7.278 | <0.001* |
| | Sampling day | | | 0.223 ± 0.153 | 1.454 | 0.155 |
| | Brood size | | | 0.046 ± 0.085 | 0.540 | 0.592 |
| GSSG | | | | | | |
| | Area | NAT | RUR | −2.928 ± 10.071 | −0.291 | 0.774 |
| | | NAT | URB | 6.910 ± 12.680 | 0.545 | 0.594 |
| | | RUR | URB | 9.838 ± 10.420 | 0.944 | 0.368 |
| | Sampling year | 2020 | 2021 | 10.558 ± 7.585 | 1.392 | 0.178 |
| | Sampling day | | | −3.412 ± 6.506 | −0.524 | 0.604 |
| | Brood size | | | 1.492 ± 3.747 | 0.398 | 0.697 |
| GSH:GSSG | | | | | | |
| | Area | NAT | RUR | 0.045 ± 0.387 | 0.116 | 0.910 |
| | | NAT | URB | −1.295 ± 0.497 | −2.607 | 0.038* |
| | | RUR | URB | −1.340 ± 0.452 | −2.963 | 0.038* |
| | Sampling year | 2020 | 2021 | −1.231 ± 0.296 | −4.160 | 0.002* |
| | Sampling day | | | 0.100 ± 0.245 | 0.408 | 0.689 |
| | Brood size | | | 0.162 ± 0.174 | 0.928 | 0.395 |
| TAS | | | | | | |
| | Area | NAT | RUR | −0.237 ± 0.785 | −0.302 | 0.765 |
| | | NAT | URB | 0.018 ± 0.987 | 0.019 | 0.985 |
| | | RUR | URB | 0.255 ± 0.780 | 0.327 | 0.745 |
| | Sampling year | 2020 | 2021 | −1.467 ± 0.617 | −2.377 | 0.022* |
| | Sampling day | | | −0.289 ± 0.539 | −0.536 | 0.595 |
| | Brood size | | | −0.140 ± 0.281 | −0.498 | 0.621 |

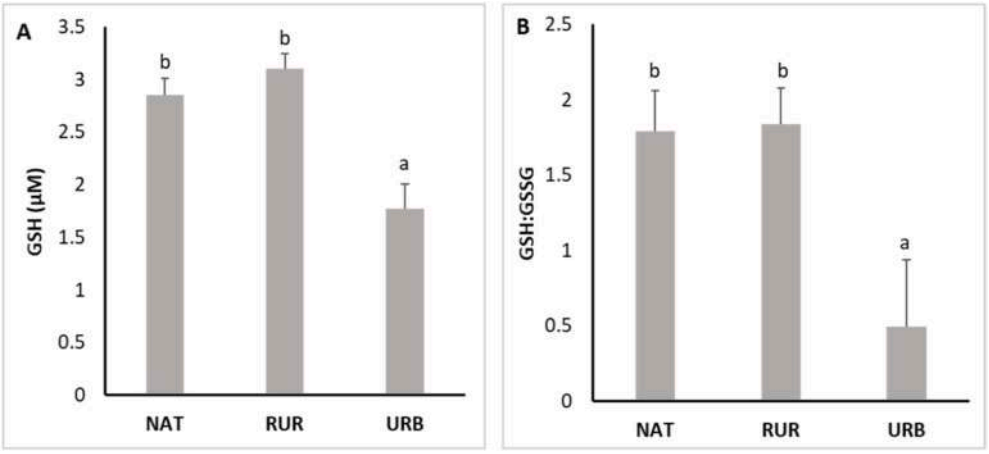


**Fig. 2.** (A) Plasma concentration of reduced glutathione (GSH) and (B) ratio between reduced and oxidized glutathione (GSH:GSSG) in common kestrel nestlings sampled in NAT = natural, RUR = rural, and URB = urban areas. Different letters indicate statistically significant differences ($p < 0.05$). Results are presented as estimated marginal means ± SE (log10-transformed values).

2021, except GSSG (Table 1). The H/L ratio, complement system activity and bactericidal capacity were higher in 2021 than in 2020; whereas the leukocyte classes and the respiratory burst did not differ between sampling years (Table 2). The comet and ENA assays were higher in 2020 than 2021 (Table 3), and a significant difference was recorded between 2021 and 2020 only for CaE (2021 higher than 2020) and not for BchE (Table 4).

The sampling day had an effect on lymphocytes number and H/L ratio. Indeed, the number of lymphocytes was higher (Estimate: 0.610 ± SE 0.239, $p = 0.025$, Table 2) in nestlings born later in the season, whilst the H/L ratio was higher in nestlings born earlier in the season (Estimate: 0.471 ± SE 0.180, $p = 0.022$, Table 2). There was also a tendency of monocytes to decrease during the season (Estimate: 0.369 ± SE 0.206, $p = 0.092$, Table 2). A marginal negative correlation also emerged between the brood size and complement system activity (Estimate: 0.613 ± SE 0.325, $p = 0.066$, Table 2). Moreover, there was a tendency for a positive covariation between brood size and ENA assay (Estimate: 16.969 ± SE 9.134, $p = 0.082$, Table 3).

## 4. Discussion

In this study we applied a blood-based multi-biomarker approach to evaluate the physiological responses of common kestrel nestlings to environments that widely differ in human activity. Briefly, we found that nestlings from URB, the area located in the centre of Rome, showed significantly lower GSH levels, slightly lower GSH:GSSG values, slightly higher numbers of monocytes, and higher inhibition of BChE activity compared to RUR. BChE activity was also significantly lower in NAT area than in RUR. Finally, common kestrels inhabiting RUR showed the highest values of DNA damage. These results were robust for any

**Table 2**
Outcomes of linear mixed models used to examine the effects of breeding areas differing in land cover and anthropogenic pressures on immune system markers in common kestrel (*Falco tinnunculus*) nestlings. *$P < 0.05$, significant difference. NAT = natural, RUR = rural, URB = urban.

| Variable | Factor | Reference Level | Level | Coefficient Estimate ±SE | *t* | *p* |
|---|---|---|---|---|---|---|
| Respiratory burst | | | | | | |
| | Area | NAT | RUR | −0.0413 ± 0.142 | −0.290 | 0.773 |
| | | NAT | URB | 0.166 ± 0.211 | 0.788 | 0.435 |
| | | RUR | URB | 0.208 ± 0.174 | 1.196 | 0.239 |
| | Sampling year | 2020 | 2021 | −0.177 ± 0.121 | −1.451 | 0.155 |
| | Sampling day | | | 0.079 ± 0.114 | 0.691 | 0.494 |
| | Brood size | | | 0.007 ± 0.067 | 0.113 | 0.911 |
| Differential WBCs count | | | | | | |
| *Heterophils* | Area | NAT | RUR | 1.810 ± 10.015 | 0.181 | 0.862 |
| | | NAT | URB | −7.135 ± 14.952 | −0.477 | 0.644 |
| | | RUR | URB | −8.945 ± 15.123 | −0.591 | 0.569 |
| | Sampling year | 2020 | 2021 | 12.335 ± 8.778 | 1.405 | 0.179 |
| | Sampling day | | | −7.356 ± 4.663 | −1.577 | 0.147 |
| | Brood size | | | −3.932 ± 4.468 | −0.880 | 0.407 |
| Differential WBCs count | | | | | | |
| *Eosinophils* | Area | NAT | RUR | 1.159 ± 0.700 | 1.657 | 0.126 |
| | | NAT | URB | 1.490 ± 1.003 | 1.485 | 0.163 |
| | | RUR | URB | 0.331 ± 1.029 | 0.322 | 0.753 |
| | Sampling year | 2020 | 2021 | 0.407 ± 0.533 | 0.763 | 0.452 |
| | Sampling day | | | 0.278 ± 0.308 | 0.905 | 0.381 |
| | Brood size | | | 0.088 ± 0.305 | 0.288 | 0.778 |
| Differential WBCs count | | | | | | |
| *Lymphocytes* | Area | NAT | RUR | −0.112 ± 0.521 | −0.214 | 0.835 |
| | | NAT | URB | 0.019 ± 0.767 | 0.025 | 0.981 |
| | | RUR | URB | 0.131 ± 10.580 | 0.168 | 0.870 |
| | Sampling year | 2020 | 2021 | −0.681 ± 0.440 | −1.546 | 0.138 |
| | Sampling day | | | 0.610 ± 0.239 | 2.553 | 0.025* |
| | Brood size | | | 0.091 ± 0.231 | 0.392 | 0.704 |
| Differential WBCs count | | | | | | |
| *Monocytes* | Area | NAT | RUR | 0.513 ± 0.423 | 1.212 | 0.255 |
| | | NAT | URB | 1.515 ± 0.632 | 2.398 | 0.032* |
| | | RUR | URB | 1.002 ± 0.630 | 1.592 | 0.138 |
| | Sampling year | 2020 | 2021 | 0.126 ± 0.371 | 0.339 | 0.738 |
| | Sampling day | | | −0.369 ± 0.206 | −1.792 | 0.092 |
| | Brood size | | | −0.053 ± 0.186 | −0.286 | 0.781 |
| H/L ratio | | | | | | |
| | Area | NAT | RUR | 0.655 ± 0.447 | −1.465 | 0.173 |
| | | NAT | URB | −0.345 ± 0.642 | −0.540 | 0.601 |
| | | RUR | URB | 0.308 ± 0.671 | 0.460 | 0.656 |
| | Sampling year | 2020 | 2021 | 0.549 ± 0.262 | 2.091 | 0.049* |
| | Sampling day | | | −0.471 ± 0.180 | −2.618 | 0.022* |
| | Brood size | | | 0.311 ± 0.191 | 1.630 | 0.132 |
| Complement activity | | | | | | |
| | Area | NAT | RUR | 0.455 ± 0.908 | 0.501 | 0.619 |
| | | NAT | URB | −1.084 ± 1.140 | −0.951 | 0.347 |
| | | RUR | URB | −1.539 ± 0.907 | −1.696 | 0.097 |
| | Sampling year | 2020 | 2021 | 1.755 ± 0.744 | 2.359 | 0.023* |
| | Sampling day | | | −0.110 ± 0.626 | −0.176 | 0.861 |
| | Brood size | | | −0.613 ± 0.325 | −1.886 | 0.066 |
| Bactericidal capacity | | | | | | |
| | Area | NAT | RUR | 0.386 ± 0.636 | 0.606 | 0.552 |
| | | NAT | URB | 0.167 ± 0.853 | 0.195 | 0.847 |
| | | RUR | URB | −0.219 ± 0.713 | −0.307 | 0.763 |
| | Sampling year | 2020 | 2021 | 1.349 ± 0.496 | 2.721 | 0.012* |
| | Sampling day | | | 0.281 ± 0.403 | 0.696 | 0.494 |
| | Brood size | | | 0.400 ± 0.258 | 1.546 | 0.143 |

variation among nests in potentially confounding variables, such as breeding date, brood size, or sampling year. Effect size estimates also showed that differences between areas were large and significant. However, our moderate sample size did not enable us to test whether differences between sites were consistent across time.

The term oxidative status refers to the amount of reactive oxygen species and antioxidants in cells or tissues. Variation of oxidative status can be evaluated by measuring the concentration of specific molecules in their oxidized and non-oxidized forms, such as reduced and oxidized glutathione, and their ratio (GSH:GSSG). It is increasingly recognized that alterations of the oxidative status can be of profound biological relevance owing to their potential impact on fitness. A number of environmental factors can affect the oxidative status of free-living animals, such as light and noise pollution, chemical contamination (e.g., organic pollutants, pesticides, heavy metals and ionizing radiation), climate change, food quality and availability (Beaulieu and Costantini, 2014). Prior studies on songbirds found that a significant reduction of the GSH:GSSG ratio in urban adults and suburban nestlings of Great tit (*Parus major*) (Isaksson et al., 2005) and in nestlings of Eurasian Blue tit (*Cyanistes caeruleus*) from industrial areas (Rainio et al., 2013). One reason for these reduced concentrations of glutathione in urban birds might lie with the exposure to heavy metals. For example, the accumulation of Pb, a redox-inactive metal that can occur at high concentrations in cities, caused a reduction of tGSH levels in Eurasian eagle-owl (*Bubo bubo*) adults and nestlings from a mining area in Spain (Espín et al., 2014). Another reason might lie with variation in parasite

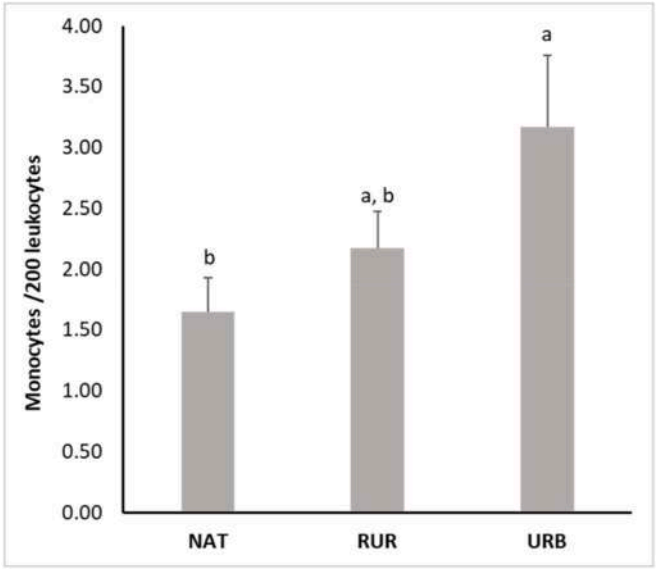


**Fig. 3.** Variation in the proportion of monocytes among the three study areas (NAT = natural, RUR = rural, and URB = urban) in common kestrel nestlings. Different letters indicate statistically significant differences ($p<0.05$). Results are presented as estimated marginal means ± SE (sqrt-root transformed values).

exposure across the natural to urban gradient because immune response can influence glutathione levels (Costantini, 2022). Conversely to our work, a prior study on kestrels carried out in central Europe did not find any significant relationship between the urban gradient and tGSH or the ratio of GSH:GSSG (Wemer et al., 2021). As with the plasma non-enzymatic antioxidant capacity (TAS), although the values found in our study were higher than those measured in other bird species (Bourgeon et al., 2012; Cohen and McGraw, 2009; Meitern et al., 2013), the three study areas showed similar values. Overall, these studies suggest that the results cannot be generalized, probably because cities can widely differ in the arrangement of land use, as well as in their contiguity with rural or natural environments outside the city. Further studies will be needed to assess which factors are responsible for the reduced levels of GSH in city birds.

The respiratory burst data did not differ among sites. This is the first study that measured the respiratory burst in Common Kestrels, so that we do not have any data to compare with. However, possible alterations of the respiratory burst process, caused by exposure to contaminants, were recorded in other bird species (Garg et al., 2004; Hansen et al., 2020). It might be that variation in phagocyte numbers across sites would influence the overall oxidative burst. However, we also found similar WBCs counts among the three study sites. The differential WBCs count is commonly applied as an indicator of immunological and infection status of individuals. Immuno-haematological alterations emerging in animals can be caused by a combined action of contaminants, a general physiological stress condition, and presence of pathogens (Jara-Carrasco et al., 2015; Latorre et al., 2016; Dean et al., 2017; Suljević et al., 2023). The H/L ratio, which is an index of stress condition in birds, owing to an increase of the number of heterophils and a decrease of lymphocytes (Davis et al., 2008) was also similar across sites, and their values were similar to those previously recorded for the kestrel (Dell'Omo et al., 2009). Monocytes are the precursors of tissue macrophages and are involved in the constitutive immune response by serving as phagocytes cells (Campbell, 1995; Vallverdú-Coll et al., 2019). Normal values of monocytes in birds vary between 0 and 3% (Gálvez Martínez et al., 2009), and their increase is considered a signal of a subacute or chronic infection and inflammation caused by bacteria or parasites (Charles-Smith et al., 2014). In our study, we found a small but significant difference in the number of monocytes among areas. We recorded slightly higher numbers of monocytes in urban nestlings than in natural nestlings with values over 3%. Moreover, the number of monocytes of urban nestlings were higher than that previously recorded in nestling kestrels (Dell'Omo et al., 2009) or in adult common kestrels clinically healthy and kept in a rescue center (values of 1.80 ± 1.48 %; (Shen et al., 2008). We can hypothesize that the high number of monocytes recorded in nestlings from URB area could be linked to a condition of infection and/or inflammation, possibly due to individuals being exposed to high levels of contamination of the Aniene River (Dell'Omo et al., 2008) or to greater parasite abundance or diversity that would cause pathogen-driven immunostimulation (Murray et al., 2019; Minias, 2023) compared to areas outside the city. For example, Nwaogu et al. (2023) found that the innate immunity of nestling African sparrowhaks *Accipiter melanoleucus* increased with urban cover. Further studies will be needed to address this hypothesis.

Nestlings from the rural site showed higher DNA damage than nestlings from the other study areas. It should be underlined that our comet test was never applied before on Common Kestrel so that basal values of comet assay for this species are still unknown. Our results are in line with those advised by the expert working group from the International Workshop on Genotoxicity Testing (Kirkland et al., 2007). Following inter-laboratory calibration, percent of DNA in the tail in control samples should be within 10–20% (Tice et al., 2000). Further investigations should be addressed to better define baseline values for Kestrels by performing *in vitro* studies on blood. The exposure of blood samples to genotoxic compounds could enable to quantify the possible contribution of chemical contaminants. Comparing our results with the literature, we can hypothesize that Common Kestrels were probably not exposed to substances and agents with genotoxic effects. DNA damage values recorded in blood of other species are higher than those measured in this study. Sicolo et al. (2010) obtained mean values of DNA tail % equal to

**Table 3**
Outcomes of linear mixed models used to examine the genotoxic effects of breeding areas differing in land cover and anthropogenic pressures in common kestrel (*Falco tinnunculus*) nestlings. *$P<0.05$, significant difference. NAT = natural, RUR = rural, URB = urban.

| Variable | Factor | Reference Level | Level | Coefficient Estimate ±SE | *t* | *p* |
|---|---|---|---|---|---|---|
| DNA tail % | | | | | | |
| | Area | NAT | RUR | 0.324 ± 0.115 | 2.811 | 0.012* |
| | | NAT | URB | −0.138 ± 0.154 | −0.900 | 0.381 |
| | | RUR | URB | −0.462 ± 0.146 | −3.172 | 0.008* |
| | Sampling year | 2020 | 2021 | −0.813 ± 0.101 | −8.012 | <0.001* |
| | Sampling day | | | 0.055 ± 0.061 | 0.901 | 0.375 |
| | Brood size | | | 0.032 ± 0.049 | 0.657 | 0.521 |
| ENAs | | | | | | |
| | | NAT | RUR | 10.009 ± 20.592 | 0.486 | 0.634 |
| | | NAT | URB | −9.876 ± 29.437 | −0.336 | 0.742 |
| | | RUR | URB | −19.886 ± 30.137 | 14.930 | 0.519 |
| | Sampling year | 2020 | 2021 | −57.988 ± 16.920 | −3.427 | 0.002* |
| | Sampling day | | | −4.920 ± 9.161 | −0.537 | 0.598 |
| | Brood size | | | 16.969 ± 9.134 | 1.858 | 0.082 |

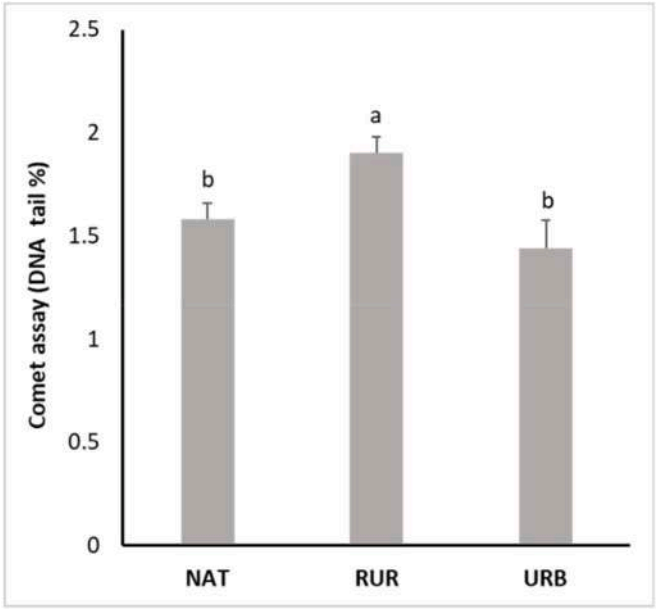


**Fig. 4.** Comet assay results of common kestrel nestlings sampled in NAT = natural, RUR = rural, and URB = urban areas. Different letters indicate statistically significant differences ($p<0.05$). Results are presented as estimated marginal means ± SE (sqrt-root transformed values).

32.49 ± 4.13 in pigeons from Milan center (Italy). Also, Bonisoli-Alquati et al. (2010) recorded a mean genetic damage with values of 68.41 ± 6.21 in barn swallows inhabiting an area with high-level of radiation. Maximum values recorded in RUR and NAT are in line with mean values (10.04 ± 4.86) of DNA tail % obtained by Bonisoli-Alquati et al. (2015) in barn swallow nestlings exposed to radiation.

The ENAs assay is an economical test and efficient tool for directly measuring DNA damage caused by environmental pollutants (De Mas et al., 2015). The ENAs assessed on Common Kestrels did not show significant differences among our study areas, although the pattern of variation was similar to that of the comet assay. This is the first study to apply this method on Kestrels; therefore, we can only make a comparison with data of other avian species sampled in areas with different anthropic impacts. The number of MN found in Common Kestrels was lower than those recorded in adult individuals of American kestrels *Falco sparverius* (19.2/10,000 or 3.84/2000) from agricultural areas (Frixione and Rodríguez-Estrella, 2020). By contrast, they were in line with those of adult birds belonging to different avian families (such as, columbidae, passeridae, turdidae, etc.) sampled in small (1.29/10,000 or 0.26/2000) and medium (2.59/10,000 or 0.52/2000) coffee farms (Souto et al., 2018). Considering urban and industrial environments, MN mean values of the natural sampling site were in line with those of adult passeriformes (0.58/10,000 or 0.12/2000) living away from urban centers, and higher than those recorded in *Sterna hirundo* nestlings (0.13/5000 or 0.052/2000) from a Tunisia site with low urban and industrial pressures (Baesse et al., 2019; Oudi et al., 2019). Moreover, MN data recorded by Baesse et al. (2019) in passeriformes living close to urban centers (1.74/10,000 or 0.35/2000) were lower than MN values that we recorded in the rural sampling site. Our MN values were similar to those shown by Oudi and collaborators (2019) in *Sterna hirundo* nestlings sampled near urban (0.50/5000 or 0.20/2000) and industrial (0.36/5000 or 0.14/2000) environments. Finally, it is important to underline that 100% of Common Kestrel individuals showed erythrocyte nuclear abnormalities and "bud" nucleus was the more abundant abnormality. In fact, our data of total ENAs were significantly higher than those showed by Frixione and Rodríguez-Estrella (2020) in *Falco sparverius* from an agricultural environment (71.5/10,000 or 14.3/2000). Therefore, the comparison with the literature showed that the specimens of RUR had MN values higher or in line with those of other avian species from urban or industrial areas. Although in RUR, sheep pasturelands and set asides are two common types of land use, the area is surrounded by several high-traffic roads, which might expose birds to potentially genotoxic factors.

To our knowledge, BChE and CaE activities have not been measured on wild Kestrels before, therefore our data are not comparable with other similar studies. Our mean values are in the range of BChE levels reported by Roy et al., 2005, for 2–3-month-old Common Kestrels (range: 0.123–0.525 μmol/min/mL) and by Oropesa et al. (2013) for *Ciconia ciconia* juveniles (0.28 ± 0.03 μmol/min/mL) housed in different recovery centers located in Europe. Moreover, the values recorded in our study are lower than those observed in adult individuals of other avian species (Dhananjayan, 2012; Fossi et al., 1996), and this is consistent with other studies that found lower values of cholinesterase activities in younger birds than in adult individuals (Roy et al., 2005). B-esterase family includes AChE, BChE and CaE enzymes. These blood hydrolases are inhibited by anticholinesterase pesticides, and some of them are involved in detoxification of OPs and CBs insecticides (Sanchez-Hernandez, 2007). The activity of BChE activity can also be modulated by factors, such as sex, age, external temperature and diet, which can determine a strong individual or species variation. Therefore, the level of B-esterase and the relative contribution of AChE and BChE to ChE activity are likely to be key factors in explaining the differential sensitivities of species to anticholinesterase insecticides. It was reported that Falconidae family had a dominant AChE contribution to the total ChE activity, while in other raptors families, BChE activity dominated (Roy et al., 2005). We found a significant reduction of BChE activity in nestlings from URB and NAT with respect to those of RUR. Contrary to our prediction, individuals of RUR did not show a reduction of BChE

**Table 4**
Outcomes of linear mixed models used to examine the neurotoxic effects of breeding areas differing in land cover and anthropogenic pressures in common kestrel (*Falco tinnunculus*) nestlings. *$P<0.05$, significant difference. NAT = natural, RUR = rural, URB = urban.

| Variable | Factor | Reference Level | Level | Coefficient Estimate ±SE | *t* | *p* |
|---|---|---|---|---|---|---|
| BChE activity | | | | | | |
| | Area | NAT | RUR | 0.092 ± 0.044 | 2.079 | 0.045* |
| | | NAT | URB | −0.048 ± 0.058 | −0.830 | 0.412 |
| | | RUR | URB | −0.140 ± 0.048 | −2.943 | 0.006* |
| | Sampling year | 2020 | 2021 | −0.024 ± 0.034 | −0.716 | 0.479 |
| | Sampling day | | | −0.004 ± 0.029 | −0.152 | 0.880 |
| | Brood size | | | −0.003 ± 0.016 | −0.177 | 0.861 |
| CaE activity | | | | | | |
| | | NAT | RUR | 10.452 ± 8.342 | 1.253 | 0.219 |
| | | NAT | URB | 12.289 ± 10.774 | 1.141 | 0.262 |
| | | RUR | URB | 1.837 ± 9.080 | 0.202 | 0.841 |
| | Sampling year | 2020 | 2021 | 25.900 ± 6.378 | 4.061 | 0.003* |
| | Sampling day | | | 1.635 ± 5.568 | 0.294 | 0.771 |
| | Brood size | | | 1.879 ± 3.128 | 0.601 | 0.552 |

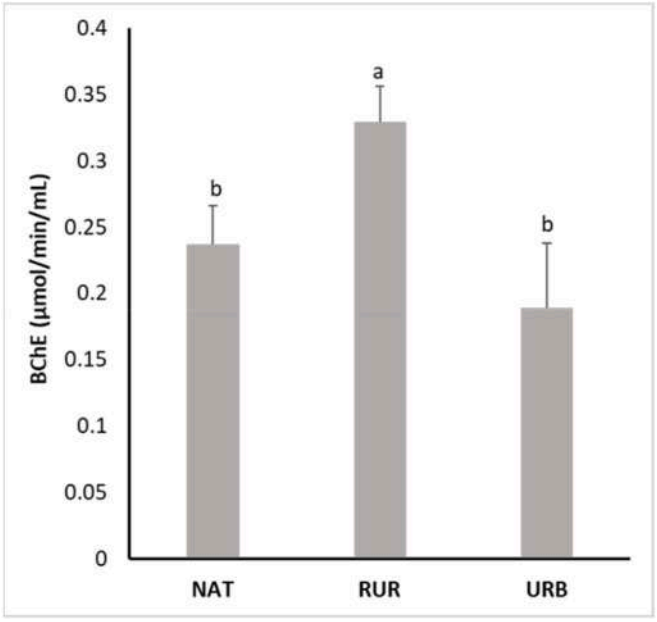


**Fig. 5.** Activity of butyrylcholinesterase (BChE) in common kestrel nestlings sampled in NAT = natural, RUR = rural, and URB = urban areas. Different letters indicate statistically significant differences ($p<0.05$). Results are presented as estimated marginal means ± SE.

activity, as in this area there are several farmlands. It might be that urban individuals were exposed to anticholinesterase compounds transported from Aniene river. In fact, as stated above, the Aniene river, crossing several areas with anthropic pressures, collects waste water that could contain different contaminants, with which individuals of common kestrels may have come into contact. Dell'Omo et al. (2008) found high concentrations of PCB congeners and of other contaminants (e.g., HCB, DDE and HEOD) in eggs collected from nests near the banks of Aniene River. BChE was found to be inhibited in other species by lipophilic contaminants, such as PAHs (Casini et al., 2006). Also, La Vigna et al. (2019) found 1,1,2-trichloroethane concentrations that exceed the legislation limit (0.2 µg/L), mainly in the eastern sector of the area, and trichloromethane concentrations that exceed the legislation limit (0.15 µg/L) in several analysed points evenly distributed in the study area.

Carboxylesterases (CaEs) are a multigene family of enzymes that hydrolyse a wide range of aliphatic/aromatic esters and choline esters, as well as some xenobiotics. They play a double role, both as detoxifying enzymes and in the defence, protecting AChE from the inactivation caused by OP and CB (Satho and Hosokawa, 1998; Caliani et al., 2021). However, CaEs can be inhibited in turn by anticholinesterase pesticides and also by heavy metals (Bjedov et al., 2023). We did not find any differences in CaE activity among study areas. Mean values of CaE activity obtained by Roy et al., 2005), on common kestrels from recovery centers were on the average of 0.53 µmoL/min/ml, which are higher than those of Common Kestrels from our study areas. Such difference is probably linked to the different CaE substrates used by us and Roy et al., 2005. Yet, we can suppose that CaE did not intervene in support of the other B-esterase enzymes.

Finally, the results of our study showed that several of the analysed biomarkers varied significantly between sampling years or with the breeding date and brood size. The between-year variation, in particular, could be related to differences in the spring temperatures and other meteorological parameters between 2020 and 2021, which might have affected prey availability and consequently nutrient and energy uptake by the birds. It might also be that the reduced human activities of 2020 due to the COVID-19 pandemics contributed to some degree to generate variation between sampling years. These results suggest that factors

**Table 5**
Effect size estimates (Hedges'g) and 95% credible intervals for the comparisons between natural (NAT), rural (RUR), and urban (URB) areas. Effect sizes are significant when the credible interval does not include zero. Significant effect sizes are shown in bold.

| BIOMARKER | AREA 1 | AREA 2 | Hedges'g | −95% | +95% |
|---|---|---|---|---|---|
| DNA tail % | NAT | RUR | -0.77 | -1.32 | -0.22 |
| DNA tail % | RUR | URB | 1.08 | 0.27 | 1.89 |
| DNA tail % | URB | NAT | −0.35 | −1.13 | 0.43 |
| ENAs | NAT | RUR | −0.13 | −0.64 | 0.38 |
| ENAs | RUR | URB | 0.25 | −0.62 | 1.11 |
| ENAs | URB | NAT | −0.14 | −0.99 | 0.72 |
| H/L ratio | NAT | RUR | 0.4 | −0.13 | 0.92 |
| H/L ratio | RUR | URB | −0.17 | −1.11 | 0.76 |
| H/L ratio | URB | NAT | −0.23 | −1.16 | 0.70 |
| Heterophils | NAT | RUR | −0.05 | −0.57 | 0.47 |
| Heterophils | RUR | URB | 0.23 | −0.7 | 1.16 |
| Heterophils | URB | NAT | −0.20 | −1.13 | 0.73 |
| Eosinophils | NAT | RUR | −0.49 | −1.02 | 0.04 |
| Eosinophils | RUR | URB | −0.13 | −1.06 | 0.80 |
| Eosinophils | URB | NAT | 0.67 | −0.27 | 1.61 |
| Lymphocytes | NAT | RUR | 0.05 | −0.47 | 0.57 |
| Lymphocytes | RUR | URB | −0.1 | −1.03 | 0.83 |
| Lymphocytes | URB | NAT | 0.05 | −0.87 | 0.98 |
| Monocytes | NAT | RUR | −0.34 | −0.87 | 0.19 |
| Monocytes | RUR | URB | −0.63 | −1.57 | 0.32 |
| **Monocytes** | **URB** | **NAT** | **1.03** | **0.07** | **2.00** |
| TAS | NAT | RUR | 0.10 | −0.52 | 0.72 |
| TAS | RUR | URB | −0.11 | −0.95 | 0.73 |
| TAS | URB | NAT | 0.01 | −0.83 | 0.84 |
| GSH | NAT | RUR | −0.40 | −1.06 | 0.27 |
| **GSH** | **RUR** | **URB** | **2.14** | **1.06** | **3.22** |
| **GSH** | **URB** | **NAT** | **−1.67** | **−2.7** | **−0.64** |
| GSSG | NAT | RUR | 0.11 | −0.55 | 0.77 |
| GSSG | RUR | URB | −0.37 | −1.27 | 0.53 |
| GSSG | URB | NAT | 0.25 | −0.65 | 1.16 |
| GSH/GSSG | NAT | RUR | −0.05 | −0.79 | 0.7 |
| **GSH/GSSG** | **RUR** | **URB** | **1.39** | **0.32** | **2.46** |
| **GSH/GSSG** | **URB** | **NAT** | **−1.31** | **−2.39** | **−0.23** |
| Respiratory burst | NAT | RUR | 0.10 | −0.51 | 0.71 |
| Respiratory burst | RUR | URB | −0.50 | −1.45 | 0.45 |
| Respiratory burst | URB | NAT | 0.41 | −0.55 | 1.37 |
| Complement activity | NAT | RUR | −0.17 | −0.77 | 0.44 |
| Complement activity | RUR | URB | 0.57 | −0.27 | 1.42 |
| Complement activity | URB | NAT | −0.40 | −1.24 | 0.44 |
| Bactericidal capacity | NAT | RUR | −0.25 | −0.98 | 0.48 |
| Bactericidal capacity | RUR | URB | 0.14 | −0.91 | 1.19 |
| Bactericidal capacity | URB | NAT | 0.10 | −0.97 | 1.17 |
| **BChE activity** | **NAT** | **RUR** | **−0.75** | **−1.42** | **−0.09** |
| **BChE activity** | **RUR** | **URB** | **1.16** | **0.16** | **2.17** |
| BChE activity | URB | NAT | −0.39 | −1.35 | 0.58 |
| CaE activity | NAT | RUR | −0.46 | −1.13 | 0.21 |
| CaE activity | RUR | URB | −0.09 | −1.04 | 0.87 |
| CaE activity | URB | NAT | 0.54 | −0.44 | 1.51 |

linked to environmental conditions during which chicks are being raised (e.g. prey availability, weather conditions) or to within-brood competition influence the physiology condition. In our models, we could control for these potentially confounding factors in order to obtain robust estimates for the comparisons among areas. However, it will be important in future studies to assess, in the long-term, whether any effect of the habitat type on the physiology of birds may also come through alterations in other environmental parameters linked to breeding biology.

## 5. Conclusions

In conclusion, we found evidence for (i) higher immune activation (monocytes), alteration of oxidative status (lower GSH and GSH:GSSG) and interference with cholinesterase activity in URB and NAT kestrels compared to RUR kestrels, and (ii) higher DNA damage in RUR kestrels compared to URB and NAT kestrels. We also found that many of our biomarkers were rather similar across the three study areas. Our multi-

biomarker approach suggests that kestrels probably face with environmental stressors that differ in typology or intensity across contrasting environments, so that concluding that cites are not adequate habitats for kestrels might be premature. Also, the between-year differences observed in various parameters suggest that conditions for each of the three habitats considered can vary across time, so that they need to be taken into account when comparing urban and non-urban populations. Given the moderate sample size, we could not test whether differences between areas were consistent across years. Thus, it will be important in future studies to assess this question. It will be also needed to better determine the main drivers of this physiological differentiation among conspecific populations breeding in different habitats, and the potential consequences for population dynamics. This information is particularly relevant to guide exploitation of lands and urban planning in a more compatible way with the health of wildlife. Finally, the three sites included in our study were located in landscape with a mosaic of land uses. Thus, it will also be very important to further replicate this work including other locations to better characterise the physiological responses of birds to different land uses.

## CRediT authorship contribution statement

**Laura Giovanetti:** Writing – original draft, Methodology, Investigation, Formal analysis. **Ilaria Caliani:** Writing – review & editing, Writing – original draft, Supervision, Methodology, Conceptualization. **Gianluca Damiani:** Investigation. **Giacomo Dell'Omo:** Writing – review & editing, Investigation. **David Costantini:** Writing – review & editing, Writing – original draft, Data curation, Conceptualization. **Silvia Casini:** Writing – review & editing, Supervision, Resources, Project administration, Conceptualization.

## Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

## Data availability

Data will be made available on request.

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## Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.envres.2024.118674.

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## 3.6. Heavy metals and POPs contaminants in feathers and eggs of Common Kestrel

The presence of contaminants (heavy metals, PCBs, OCPs, PBDEs and PCDD/Fs) on non-lethal biological material (unhatched/abandoned eggs and feathers) was evaluated to integrate chemical analysis information with blood biomarkers responses and to investigate how the different environments can influence birds' physiology to get a more complete picture of the Common Kestrels state of health.

### 3.6.1. Material and methods

#### 3.6.1.1. Study areas

This research was conducted in three different sites in the countryside and in the city of Rome within the Lazio Region (Italy). These areas, namely Castel di Guido, Decima Malafede, and Aniene Park, are characterized by different land cover and anthropogenic impacts.

Details on the features of the study areas have been described above (see material and methods of paragraph 3.5).

#### 3.6.1.2. Feathers and eggs collection

The two sampling campaigns were conducted between May and June in 2020 and 2021. Specifically, feathers were sampled in 2020, while eggs were collected both in 2020 and 2021 seasons. All the biological materials were sampled from nest boxes located on TERNA s.ps. power lines in the study areas.

The feathers were plucked from the breast of 41 nestlings (10 nests) after carrying out the blood sampling. Feathers were stored at room temperature in paper bags until heavy metal analysis.

A total of 20 unhatched or abandoned eggs were collected from 7 nests during their routine inspection. Eggs were found and sampled in two of the study areas, namely Castel di Guido and Aniene Park. They were stored until further laboratory processing and analysis of persistent organic pollutants (POPs).

#### 3.6.1.3. Analytical methods

**Analysis of heavy metals in feathers**

Feathers from pullets belonging to the same nest were merged (pool) and washed several times with distilled water to get rid of potential superficial contamination (Figure 3.13). Then the feathers were left to dry under hood, covered with clean filter paper. At the time of analysis, they were

homogenized by shredding. Approximately 0.1 g of the homogenized samples were placed in Teflon containers and 2 ml of nitric acid ($HNO_3$) and 0.5 ml of hydrogen peroxide ($H_2O_2$) was added. The containers were closed in a stain steel block and heated at temperature of 160 °C for 10 hours, to complete the disintegration of organic matter. The obtained solutions were then transferred into polyethylene test tubes and made up to volume of 10 mL with ultrapure water and used for the analytical determination of heavy metals with atomic absorption spectrometers. In detail, Hg analysis was performed using cold vapor technique with a spectrometer Perkin Elmer Mod. FIMS 400 (Flow Injection Mercury System) and Cd, Cu and Pb were performed using Graphite Furnace technique with a spectrometer Analytik Jena Mod. ContrAA 700. To verify any contamination of reagents and the accuracy of the results, in each analysis batch a blank solution and standard references materials were always run. To obtain the calibration curve, internal additions method was used: standard solutions at increasing known concentrations of the analysed element were added to a sample replicate. The final result was expressed as mg/kg dry weight (dw).

**Figure 3.13.** Common Krestel feathers after wash with distilled water.

**Analysis of POPs in eggs**

*Chemical Treatment and Analysis of Samples*

The eggs were stored at -80ºC until processing. Once thawed, they were homogenized and lyophilized to remove their aqueous content (Figure 3.14). Throughout all stages related to the analytical treatment of the egg samples, the materials used (glass, plastic, or metal) were thoroughly cleaned before use. They were soaked overnight in Nuclean soap (National Diagnostics, Georgia,

USA, specialized for the removal of isotopically labeled compounds), then rinsed, dried with acetone, and washed thrice with each of the following solvents in decreasing order of polarity: acetone, dichloromethane, and n-hexane.

*Extraction*

For extracting the target analytes, a protocol employing ultrasonication was followed. Approximately 0.2 g of lyophilized egg material was homogenized with 1.5 g of anhydrous sodium sulfate in a mortar and introduced into a 25 mL cylindrical flask. Then, they were fortified with a known amount of isotopically labeled compounds. Specifically 20 µL of a solution containing 18 congeners of 13C12-PCB, 10 µL of a solution containing 26 congeners of 13C12-PBDEs, 10 µL of a solution containing 17 congeners of 13C12-PCDD/F, and 15 µL of 13C-organochlorine pesticides were added (namely, 13C6-PeCB, 13C6-HxCB, 13C6-α-HCH, 13C6-ɣ-HCH, 13C10-p,p-DDE, 13C10-o,p'-DDT, 13C10- p,p'-DDT, 13C9-α-endosulfan and 13C9-β-endosulfan).

After fortification, 10 mL of a cyclohexane:acetone mixture (3:1, v/v) was added. The flask containing the solvent and sample underwent sonication in an ultrasonic bath (J.P. Selecta, Barcelona, Spain) at room temperature for 15 minutes. This sonication process was repeated three times, changing the solvent between each cycle, yielding a final volume of approximately 30 mL in a 200 mL glass cell of a TurboVap® evaporation system (Zynmarck Inc., Hopkinton, Massachusetts, USA). Subsequently, the extract was concentrated to a final volume of around 1 mL using nitrogen at 0.8 bars and a temperature of 40°C in the TurboVap® system. The final volume was transferred to a 15 mL Falcon tube along with a wash of the TurboVap® cell performed with cyclohexane:ethyl acetate (1:1, v/v) and brought up to 5 mL using the same solvent mixture. Samples were centrifuged at 2000 rpm for 5 min at room temperature, and the supernatant was transferred to a 15 mL cylindrical glass cell.

*Purification*

The purification stage aimed to separate lipids and/or impurities co-extracted with the target analytes in the previous step. To ensure maximal removal of undesired compounds, two distinct purification procedures were conducted for each sample. The first utilized the GPCuno system (LCTech GmbH, Dorfen, Germany), an automatic gel permeation chromatography system (GPC). The elution of the sample employed a cyclohexane:ethyl acetate solvent mixture (1:1, v/v), injecting the 5 ml of the extract obtained in the previous step into the equipment using a glass syringe. The process lasted 55 min, collecting three different fractions. Only the first two fractions were collected

in TurboVap® cells. Fraction I (110mL) contained lipids and larger molecules, fraction II (110 ml) contained the target analytes, and fraction III (40 mL) corresponded to column cleaning and was discarded. Both fractions (I and II) were concentrated to 1 mL using the TurboVap® system. The milliliter from fraction II was transferred to a beaker along with two washes of the cell using n-hexane:dichloromethane (9:1, v/v) and brought up to a volume of 4 mL using the same mixture, which was then divided into two aliquots. One mL was collected in a vial (previously heated at 400°C to ensure the absence of any target analytes), used for pesticide content analysis. The other 3 ml were collected in a TurboVap® vessel and concentrated to 1 mL. This resulting milliliter from the second aliquot underwent re-purification by adsorption chromatography using a 10 mL glass column, packed with 1.5 g of 44% weight-modified acidic silica with sulfuric acid and an approximate amount of granular anhydrous sulfate both below and above the silica. After conditioning the column with 8 mL of n-hexane:dichloromethane (9:1, v/v), the sample was loaded and eluted with 10 mL of the same solvent mixture. The purified eluate was collected in a TurboVap® cell, concentrated to 1 mL, and then transferred to a vial. As a result, each sample yielded two vials vials, each containing 1 mL. One was used to measure the pesticide content, and the other for PCB, PBDE, and PCDD/F content analysis. Additionally, the fraction containing lipid content was collected in a pre-weighed topaz vial using a precision balance.

*Concentration*

The contents of the vials were evaporated to dryness using nitrogen at 40°C with a Pasvial apparatus (Model V3, HiTC. S.A., Spain). Once dried, the vials were reconstituted with a mixture of 13C isotopically labeled standards as injection standards.

For the aliquot used to analyze pesticides, a volume made up of 10 µL of PCB IS and 10 µL of n-nonane was used. For the aliquot used to analyze the rest of POPs, 10 µL of PCB IS, 5 µL of PBDE IS, and 5 µL of PCDD/F IS were added. After reconstitution, the vials were vortexed and stored in a freezer until instrumental analysis. Simultaneously, the vial with fraction I was concentrated using Pasvial until complete solvent evaporation. It was then placed in an oven at 105°C for 30 min. After cooling to room temperature overnight, the vial was reweighed on a precision balance to determine the weight of the extracted organic matter, representing the lipid content of the samples.

*Instrumental Analysis*

Identification and quantification of 8 mono-ortho substituted PCBs (105, 114, 118, 123, 156, 157, 167, and 189), 4 non-ortho substituted PCBs (77, 81, 126, and 169), 6 indicator PCBs (28, 52, 101,

138, 153, 180), 17 congeners 2,3,7,8-substituted of PCDD/Fs, and 26 PBDE congeners were performed using gas chromatography (GC) coupled with high-resolution mass spectrometry (HRMS) on a Trace GC Ultra chromatograph (Thermo Fisher Scientific, Milan, Italy) connected to a DFS magnetic sector mass spectrometer (Thermo Fisher Scientific, Bremen, Germany). 1 µL of each extract was injected in splitless mode at 260 °C using helium as the carrier gas. PBDE separation used a Rxi-5Sil ms column (dimensions 15m x 0.25 mm x 0.10 µm, Restek, USA), while PCB and PCDD/F separation used a DB-5ms column (dimensions 60m x 0.25 mm x 0.25 µm Agilent J&W, USA). The mass spectrometer used an electron impact source (at 45 eV) for analyte ionization, operated in selected ion monitoring (SIM) mode with two major ions for each analyte and a resolution of 10000 at 10% valley height. Analyte quantification was carried out using the isotopic dilution method. Detailed instrumental parameters can be found in previous works (Bartalini et al., 2019; Morales et al., 2022). Identification and quantification of organochlorine pesticides (PeCB, HCB, p,p'-DDT, o,p'-DDT, p,p'-DDE, o,p'-DDE, p,p'-DDD, o,p'-DDD, α-HCH, β-HCH, γ-HCH, α-endosulfan, and β-endosulfan) were carried out using GC coupled to a low-resolution tandem mass spectrometer with a triple quadrupole analyzer (GC-QqQ-MS/MS). Helium was used as carrier gas. The gas chromatograph used was a 7890B (Agilent, Palo Alto, CA, USA) equipped with a programmable temperature vaporization injector (PTV) operating in splitless mode and a DB35-ms column (dimensions 30 m x 0.25mm x 0.25 µm, Agilent J&W, USA). The mass spectrometer was a 7010B (Agilent, Palo Alto, CA, USA) equipped with a high-efficiency 70eV electron impact ionization source. A source temperature of 260°C and quadrupole temperatures of 150°C were used. A volume of 1 µL was injected, and the multiple reaction monitoring (MRM) mode was employed, specifically monitoring two transitions for each analyte.


**Figure 3.14.** Common kestrel eggs (a) and egg content after freeze-drying process (b).

#### 3.6.1.4. Statistical analysis

The heavy metals data obtained by the feathers analysis were processed by statistical analysis using the non-parametric test Kruskal-Wallis to highlight the differences between groups. Correlations between biomarkers and heavy metals were also investigated. Statistical analysis were conducted through the free software Past4.03. Means and standard deviations have been calculated with Microsoft excel.

The POPs data obtained by the eggs analysis were processed by statistical analysis using R-4.3.1 software (www.r-project.org). Logarithmic transformation was applied to the data prior to analysis. Data and derived variables exhibited non-normal distribution (Shapiro-Wilk test, $p<0.05$). Wilcoxon rank sum test ($p<0.05$; Table 3.8), Yuen's test on trimmed means ($p<0.05$; Table 3.9), and Asymptotic Two-Sample Brown-Mood Median Test ($p<0.05$; Table 3.10) were utilized to assess differences in concentrations among locations.

### 3.6.2. Results and discussion

#### 3.6.2.1. Heavy metals levels in feathers

In Table 3.6 mean ± SD and range values of heavy metals (Cd, Pb, Hg and Cu) detected in feathers of common kestrels from the present study and, for comparative purposes, also from other studies conducted worldwide, with different anthropic impacts, are reported. As mentioned above, feathers were collected in the three study areas (Castel di Guido, Decima Malafede and Aniene Park) during 2020. The concentration of Cd and Pb were higher in feathers of individuals from Castel di Guido site, while Hg and Cu were higher in individuals from the Aniene Park. However, these differences were not statistically significant. Although Hg from Aniene Park showed mean values about double and Pb about halved than those found in Castel di Guido and Decima Malafede, the lack of statistical significance found can be due to the relatively small sample size for each area and to a high intraspecies difference emerged in heavy metal results.

Indeed, these results should be confirmed by further investigation involving a larger sample size for each area, nevertheless, they could provide a preliminary indication of possible trends o particular situations. Specimens from Aniene Park area, even though located within the urban perimeter, may be affected by the presence of a river ecosystem while specimens from Castel di Guido and Decima Malafede areas, live in the countryside characterized by terrestrial ecosystems. The higher Hg and lower Pb concentration in specimens from Aniene Park could be related to a different input through the diet with prey linked to the aquatic trophic web.

**Table 3.6.** Mean values ± SD and the range (in brackets) of Cd, Pb, Hg, and Cu levels detected in common kestrel feathers from our and other studies. Values are expressed as mg/kg dw. Feather types: BD: breast down; T: tail, P: primary; B: breast; S: secondary; C: covert. Other singles indicate: nd= values below the limit of detection, *ns*= not specified; M= male, F= female. The ***** indicate that individuals arrived from recovery centers.

| **Species** | **Element** | | | | **Location** | **Sample origin** | **Feather type** | **Life stage** | **Sex** | **Reference** |
|---|---|---|---|---|---|---|---|---|---|---|
| | **Cd** | **Pb** | **Hg** | **Cu** | | | | | | |
| Common kestrel | 0.05±0.02<br>(0.03-0.08)<br>0.04±0.02<br>(0.03-0.06)<br>0.03<br>– | 2.57±2.09<br>(0.47-6.08)<br>2.17±0.72<br>(1.14-2.68)<br>1.57<br>– | 0.45±0.14<br>(0.34-0.68)<br>0.57±0.21<br>(0.27-0.72)<br>1.23<br>– | 13.22±6.64<br>(9.37-24.98)<br>9.69±1.37<br>(7.84-10.78)<br>13.69<br>– | Italy | Countryside<br>(Castel di Guido)<br>Countryside<br>(Decima Malafede)<br>Urban<br>(Aniene Park) | BD | Nestlings | *ns* | Present study |
| Common kestrel | – | 0.042±0.016<br>(0.015-0.128) | – | 2.117±0.250<br>(0.145-3.045) | Iran | Urban * | T | Juveniles, adults | *ns* | Manvaldi and Shamabadi, 2022 |
| Common kestrel | –<br>– | –<br>– | 0.36±2.25<br>(0.00-1.33)<br>0.42±0.31<br>(0.09-1.51) | –<br>– | China | Industrial | T<br>P | *ns* | *ns* | Ma et al., 2021 |
| Common kestrel | 0.06±0.16<br>(nd-1.002) | 2.51±1.56<br>(0.42-7.65) | – | 10.1±6.20<br>(4.46-43.3) | Spain | Doñana Natural Park-Guadalquivir estuary<br>Interested by a mining spill disaster and increase of urban, industrial and agricultural activities. | B | > 2 years | M, F | Manzano et al., 2021 |
| Common kestrel | 0.06±0.08<br>– | 4.35±20.65<br>– | – | 11.82±7.77<br>– | Spain | Different areas from Tenerife Ireland | T | Juveniles, adults | M, F | Rodríguez-Álvarez et al., 2021 |

| | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| Common kestrel | 0.20±0.18<br>– | 2.10±1.57<br>– | 0.59±0.36<br>– | – | Hungary | Mountainous, woody habitats, steppe, sand-hills and rivers * | P, S, C | Juveniles, adults | M, F | Grúz et al., 2019 |
| Common kestrel | 0.94±0.07<br>(0.80-1.03) | 2.94±0.38<br>(2.21-3.51) | – | 4.43±0.39<br>(3.95-5.21) | Pakistan | Industrial<br>Agricultural | *ns* | *ns* | *ns* | Abbasi et al., 2015 |
| Common kestrel | | | 1.43±0.34<br>2.01±0.17 | | Iran | Museum | S<br>T | *ns* | *ns* | Zolfaghari et al., 2007 |
| Common kestrel | | | 2.9±3.0 | | Finland | Museum | *ns* | Juveniles, adults | M, F | Lodenius and Kuusela, 1985 |

The metals most commonly associated with poisoning of humans and animals are arsenic (As), Cd, Pb and Hg. Exposure to these contaminants can occur due to air and water pollution, contaminated food and ingestion of Pb based paints (Grúz et al., 2019). Birds can be exposed to heavy metals both by physical contact and by consumption of contaminated food (Licata et al., 2010; Lodenius and Solonen, 2013). Heavy metal level can be evaluated in several organs and tissues (such as, liver, kidney, muscle and bone) but also in feathers, eggs and excreta. The use of feathers has many advantages such as easy sampling and repeatability, if necessary, without compromising the health of the animal (Adout et al., 2007). Feathers reflect the levels of these elements in the blood at the time of their growth. Indeed, during this phase, feathers are connected with blood vessels and metals present in blood (ingested through contaminated food or mobilized by organs) can accumulate in the feathers binding to thiol groups of keratin (Dauwe et al. 2003; Manjula et al., 2015). The presence of metals in feathers represent the local exposure to environmental contamination (Burger, 1993; Burger, 1996); however, it is essential to consider that age, sex, habitat modification, or diet during the moulting period can influence the concentration of these elements (Manzano et al., 2021).

Cd is a highly toxic element that can have detrimental effects on the endocrine system, kidney function, reproduction, and growth of birds. It can also compete with essential elements, leading to their deficiencies (Grúz et al., 2019; Kaur and Khera, 2018). The levels of Cd that we found in feathers of common kestrels are in line with those recorded by Manzano and collaborators (2021) in museum specimens and wild individuals of Guadalquivir estuary. Even though, this site was interested by a mining spill disaster occurred in 1998, Cd, differently from other elements, did not show significant temporal trends in kestrel feathers. Our values of Cd are also consistent with those recorded by Rodríguez-Álvarez et al. (2021) in individuals from the Tenerife Island where Industrial activity is primarily composed of small and medium-sized businesses, but, in the city of Santa Cruz de Tenerife (where most of the individuals were sampled), is also present an oil refinery. We can suppose that Cd contamination, differently from other elements, likely does not occur in that areas and/or common kestrel feathers does not record the concentration variations for this element. Within the Cd levels of these studies areas, likely homeostasis mechanisms can be effective in sequestering this element in different tissue than feathers. After uptake through the food intake as a major route of exposure,

Cd enters in the blood circulation and reach target organs such as liver and kidneys, where it can be complexed by metallothioneins, low molecular weight and sulphydryl-rich proteins acting as detoxification agent (Scheuhammer, 1987). On the other hand, our results are lower than those reported by other studies conducted in Hungary with no specific description of anthropogenic impacts (Grúz et al., 2019), and lower than those from areas in Pakistan with intensive agricultural and industrial areas (Abbasi et al., 2015). This latter study, showing extremely higher Cd level (exceeding one order of magnitude) compared to the present research, highlights the increasing human population, urbanization, and excessive industrial and agricultural practices responsible for high Cd levels in the environment and the high accumulation of this element in avian feathers also from different bird species all over Pakistan.

At levels varying from 0.1 to 2 mg/kg in plumage some toxic impacts of Cd towards seabirds have been noted (Burger and Gochfeld, 2000) and 2 mg/kg in feathers has been indicated as general toxicity thresholds value for Cd. We can therefore suppose that common kestrel from our study areas did not experience severe effects caused by Cd presence.

Similar to other metals, Pb have the ability to bind to the ionic sites of proteins, competing with other elements such as zinc and calcium and compromising proper cellular function and survival. This element can affect multiple organs and organ systems (liver, renal system, immune function, nervous system) (Williams, 2017). It is noteworthy to underlines that, despite legal restrictions on the use of Pb in fuel production, traces of this element are still found in the environment, often associated to the hunting activities. This type of activity, today represent the most significant and widespread source of Pb affecting wild animals (Romero et al., 2019), in Italy it is regulated for wetlands and for Special Protection Areas only.

Levels of Pb found in the present study, especially in Castel di Guido and Decima Malafede areas, are in line with those recorded in common kestrel by Grúz and collaborators (2019) in Hungary; however, no specific descriptions of anthropogenic impacts are available for this study because specimens of a bird hospital center were considered. Similar Pb values have been also found in areas subject to various anthropogenic impacts namely the Guadalquivir estuary, with mining spill disaster

occurred in 1998 and recent increase of other human impacts (Manzano et al., 2021), and the heavily industrialized areas in Pakistan (Abbasi et al., 2015).

Our Pb data appear higher only compared to those emerged in common kestrel from urban and agricultural areas of Iran (Manvaldi and Shamabadi, 2022). Moreover, the overall data from our study areas exhibit Pb feather levels in common kestrel that are below those known to cause sublethal behavioral or reproductive effects; in fact, Pb concentrations above the toxicity thresholds of 4 mg/kg in feathers are associated with delayed parental and sibling recognition, impaired thermoregulation, locomotion, depth perception and feeding behaviour, and lowered chick survival (Franson, 1996; Burger and Gochfeld, 2000).

Mercury exposure in birds wasfound to have a wide range of toxic effects, such as, neurotoxics alterations, reduced reproductive output, impairment of immune function, and behavioural alteration (Whitney and Cristol, 2017). Concentrations of this element in the feathers of common kestrel nestlings from Castel di Guido and Decima Malafede are in line with levels shown in individuals of the same species from industrial areas in China (Ma et al., 2021). On the other hand, our values recorded in individuals from Aniene Park, are three times higher than those of Ma et al. (2021) above mentioned and the double of those reported by Grúz and collaborators (2019) in specimens of a bird hospital center with no specific descriptions of anthropogenic impacts.

Comparing our results among the different areas considered, Hg concentration in specimens from Aniene Park could be related to a different input through the diet with prey linked to the aquatic trophic web with Hg possibly higher than those linked to terrestrial such as that reported in the mentioned literature studies (Grúz et al., 2019; Ma et al., 2021). However, the values we recorded in all study areas are lower than those indicated in the literature studies (5 mg/kg) as associated with sublethal effects, namely reproductive deficits such as lower clutch size and egg size, lower hatching rate, decreased chick survival and overall decreased reproductive success (Eisler, 1987; Furness et al., 1989; Furness 1996; Burger and Gochfeld, 2000).

Copper, differently from Cd, Pb and Hg, is an essential trace element involved in several physiological processes and functions especially in most oxidation-reduction processes where it acts as a coenzyme as well as in the formation of bone tissue etc. However, if Cu is present in high concentrations, it can cause adverse effects also on birds

(Kalisińska, 2019). High concentrations and chronic exposure can cause serious harmful effects such as reproductive, respiratory, hematological, hepatic gastrointestinal, endocrine, and ocular damage as well as causing cancer (Abdullah et al. 2015). Cu levels emerged in common kestrels from our study areas are consistent with those of 2 years old kestrels from the Guadalquivir estuary (Manzano et al., 2021) and those from different site of the Tenerife Island characterized by the presence of small business and a refinery plants (Rodríguez-Álvarez et al., 2021). However, unexpectedly, our values are higher than those emerged in kestrels from urban areas with strong anthropic impacts in Iran (Manvaldi and Shamabadi, 2022) and those from heavily industrialized areas in Pakistan (Abbasi et al., 2015). This may be likely related to the specificity of the anthropic activity carried out in these mentioned studies with a possible low use of Cu compared to other elements, namely urban areas (Manvaldi and Shamabadi, 2022) and industrialized district (Abbasi et al., 2015). Differently, in our investigated areas, mainly located in the countryside, agriculture treatments may be responsible for a large Cu input and affects the Cu levels in specimens' feathers.

The literature lacks data on baseline Cu levels in feathers for avian species. Additionally, there is an absence of established threshold levels for copper toxicity in terrestrial animals, birds included. The proposed physiological copper ranges in organs and tissues often vary significantly. Indeed, as reported by Kalisińska (2019), the variations observed of copper concentrations are influenced by metabolic functions (Cu is an essential trace element involved in various metabolic functions), homeostatic regulation, copper-binding proteins (such as ceruloplasmin and metallothioneins) and environmental exposure from dietary sources or environmental contamination.

### 3.6.2.2. POPs levels in eggs

Persistent organic pollutions' concentration data were expressed in ng/g ww. In the present study, detected ∑POPs concentrations ranged from 17.4 to 975 ng/g ww, with a median of 469 ng/g ww (Table 3.7 and Figure 3.15). Statistical analysis, including the Wilcoxon rank-sum test, Yuen's test on trimmed means, and the Asymptotic Two-Sample Brown-Mood Median Test, indicates significantly higher median values for both ∑POPs and all target families in Aniene Park compared to Castel di Guido ($p < 0.05$). Notably, in falcon eggs from both locations, the relative abundance based on median concentrations followed this order: ∑PCBs > ∑OCPs > ∑PBDEs > ∑PCDD/Fs (Figure 3.16).

**Table 3.7.** Mean, Median and range values of POPs concentration (ng/g ww) detected in common kestrel eggs from the study areas (Castel di Guido and Aniene Park). n indicates the sample size.

| Study area | Overall (n=20) | | | | Castel di Guido (n=9) | | | | Aniene Park (n=11) | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| ng/g ww | Mean | Median | Min | Max | Mean | Median | Min | Max | Mean | Median | Min | Max |
| **PCB-28** | 0.596 | 0.674 | 0.000 | 1.252 | 0.213 | 0.202 | 0.000 | 0.670 | 0.910 | 0.882 | 0.636 | 1.252 |
| **PCB-52** | 0.209 | 0.044 | 0.000 | 1.266 | 0.257 | 0.007 | 0.000 | 1.266 | 0.170 | 0.052 | 0.000 | 0.762 |
| **PCB-77** | 1.392 | 1.654 | 0.052 | 3.371 | 0.151 | 0.129 | 0.052 | 0.310 | 2.407 | 2.339 | 1.496 | 3.371 |
| **PCB-81** | 0.074 | 0.088 | 0.001 | 0.183 | 0.010 | 0.011 | 0.001 | 0.020 | 0.127 | 0.130 | 0.080 | 0.183 |
| **PCB-101** | 0.298 | 0.109 | 0.000 | 1.853 | 0.350 | 0.084 | 0.000 | 1.853 | 0.256 | 0.162 | 0.000 | 1.474 |
| **PCB-105** | 27.466 | 31.522 | 0.349 | 61.949 | 5.489 | 0.918 | 0.349 | 18.199 | 45.447 | 46.185 | 31.228 | 61.949 |
| **PCB-114** | 1.628 | 1.851 | 0.032 | 3.080 | 0.412 | 0.070 | 0.032 | 1.286 | 2.624 | 2.737 | 1.845 | 3.080 |
| **PCB-118** | 109.701 | 112.121 | 1.130 | 246.430 | 28.314 | 3.177 | 1.130 | 95.203 | 176.291 | 180.150 | 100.607 | 246.430 |
| **PCB-123** | 0.925 | 0.772 | 0.012 | 2.866 | 0.185 | 0.043 | 0.012 | 0.496 | 1.531 | 1.277 | 0.610 | 2.866 |
| **PCB-126** | 0.926 | 0.300 | 0.000 | 3.133 | 0.149 | 0.046 | 0.000 | 0.529 | 1.561 | 1.818 | 0.000 | 3.133 |
| **PCB-138** | 23.155 | 24.473 | 0.494 | 52.679 | 6.046 | 1.176 | 0.494 | 20.705 | 37.153 | 37.272 | 19.157 | 52.679 |
| **PCB-153** | 54.074 | 64.008 | 1.578 | 120.764 | 18.491 | 2.830 | 1.578 | 63.197 | 83.187 | 80.550 | 41.565 | 120.764 |
| **PCB-156** | 19.562 | 22.016 | 0.431 | 40.934 | 5.408 | 0.624 | 0.431 | 18.670 | 31.143 | 32.340 | 17.768 | 40.934 |
| **PCB-157** | 4.076 | 4.181 | 0.101 | 8.739 | 1.103 | 0.150 | 0.101 | 3.449 | 6.508 | 6.833 | 3.908 | 8.739 |
| **PCB-167** | 21.980 | 24.393 | 0.645 | 46.225 | 6.472 | 0.951 | 0.645 | 20.445 | 34.669 | 37.037 | 22.021 | 46.225 |
| **PCB-169** | 0.048 | 0.034 | 0.000 | 0.142 | 0.013 | 0.006 | 0.000 | 0.036 | 0.077 | 0.073 | 0.000 | 0.142 |
| **PCB-180** | 20.207 | 20.599 | 0.775 | 47.520 | 4.892 | 1.418 | 0.775 | 14.020 | 32.738 | 34.960 | 16.307 | 47.520 |
| **PCB-189** | 1.851 | 1.837 | 0.081 | 4.923 | 0.436 | 0.117 | 0.081 | 1.318 | 3.008 | 3.102 | 1.485 | 4.923 |
| **∑PCBs** | **288.169** | **321.831** | **6.114** | **576.734** | **78.390** | **14.046** | **6.114** | **258.085** | **459.805** | **491.447** | **263.280** | **576.734** |

| | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **PBDE-7** | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 |
| **PBDE-15** | 0.0014 | 0.0000 | 0.0000 | 0.0164 | 0.0014 | 0.0000 | 0.0000 | 0.0125 | 0.0015 | 0.0000 | 0.0000 | 0.0164 |
| **PBDE-17** | 0.0005 | 0.0003 | 0.0000 | 0.0030 | 0.0005 | 0.0004 | 0.0000 | 0.0016 | 0.0005 | 0.0000 | 0.0000 | 0.0030 |
| **PBDE-28** | 0.0016 | 0.0005 | 0.0000 | 0.0069 | 0.0021 | 0.0011 | 0.0000 | 0.0069 | 0.0012 | 0.0000 | 0.0000 | 0.0044 |
| **PBDE-47** | 0.0916 | 0.0639 | 0.0000 | 0.3265 | 0.0281 | 0.0108 | 0.0000 | 0.1203 | 0.1435 | 0.1143 | 0.0000 | 0.3265 |
| **PBDE-49** | 0.0125 | 0.0110 | 0.0000 | 0.0389 | 0.0072 | 0.0072 | 0.0000 | 0.0150 | 0.0169 | 0.0143 | 0.0000 | 0.0389 |
| **PBDE-66** | 0.0004 | 0.0000 | 0.0000 | 0.0036 | 0.0008 | 0.0000 | 0.0000 | 0.0036 | 0.0002 | 0.0000 | 0.0000 | 0.0014 |
| **PBDE-71** | 0.0022 | 0.0018 | 0.0000 | 0.0107 | 0.0010 | 0.0005 | 0.0000 | 0.0029 | 0.0032 | 0.0025 | 0.0000 | 0.0107 |
| **PBDE-77** | 0.0000 | 0.0000 | 0.0000 | 0.0000 | 0.0000 | 0.0000 | 0.0000 | 0.0000 | 0.0000 | 0.0000 | 0.0000 | 0.0000 |
| **PBDE-85** | 0.0333 | 0.0049 | 0.0000 | 0.2938 | 0.0002 | 0.0000 | 0.0000 | 0.0009 | 0.0605 | 0.0142 | 0.0000 | 0.2938 |
| **PBDE-99** | 1.3327 | 0.4066 | 0.0000 | 8.1063 | 0.0410 | 0.0287 | 0.0000 | 0.0904 | 2.3896 | 1.0616 | 0.0000 | 8.1063 |
| **PBDE-100** | 0.7559 | 0.2614 | 0.0000 | 4.2740 | 0.0303 | 0.0223 | 0.0000 | 0.0609 | 1.3496 | 0.6769 | 0.0000 | 4.2740 |
| **PBDE-119** | 0.0050 | 0.0000 | 0.0000 | 0.0222 | 0.0011 | 0.0000 | 0.0000 | 0.0054 | 0.0082 | 0.0000 | 0.0000 | 0.0222 |
| **PBDE-126** | 0.0010 | 0.0000 | 0.0000 | 0.0125 | 0.0023 | 0.0000 | 0.0000 | 0.0125 | 0.0000 | 0.0000 | 0.0000 | 0.0000 |
| **PBDE-138** | 0.0745 | 0.0289 | 0.0000 | 0.4663 | 0.0021 | 0.0000 | 0.0000 | 0.0072 | 0.1336 | 0.0833 | 0.0000 | 0.4663 |
| **PBDE-153** | 3.0196 | 1.9708 | 0.0000 | 10.3617 | 0.3389 | 0.1605 | 0.0000 | 0.9202 | 5.2129 | 5.4373 | 0.0000 | 10.3617 |
| **PBDE-154** | 0.3079 | 0.1708 | 0.0000 | 1.6445 | 0.0394 | 0.0291 | 0.0000 | 0.0963 | 0.5276 | 0.3968 | 0.0000 | 1.6445 |
| **PBDE-156** | 0.0000 | 0.0000 | 0.0000 | 0.0000 | 0.0000 | 0.0000 | 0.0000 | 0.0000 | 0.0000 | 0.0000 | 0.0000 | 0.0000 |
| **PBDE-183** | 0.456 | 0.281 | 0.000 | 1.442 | 0.071 | 0.063 | 0.000 | 0.152 | 0.771 | 0.742 | 0.000 | 1.442 |
| **PBDE-184** | 0.016 | 0.003 | 0.000 | 0.116 | 0.029 | 0.000 | 0.000 | 0.116 | 0.005 | 0.005 | 0.000 | 0.014 |
| **PBDE-191** | 0.003 | 0.000 | 0.000 | 0.014 | 0.002 | 0.000 | 0.000 | 0.014 | 0.005 | 0.003 | 0.000 | 0.013 |
| **PBDE-196** | 0.212 | 0.108 | 0.000 | 0.787 | 0.024 | 0.019 | 0.000 | 0.085 | 0.366 | 0.267 | 0.000 | 0.787 |
| **PBDE-197** | 0.136 | 0.077 | 0.000 | 0.470 | 0.019 | 0.014 | 0.000 | 0.053 | 0.233 | 0.189 | 0.000 | 0.470 |
| **PBDE-206** | 0.183 | 0.147 | 0.000 | 0.638 | 0.046 | 0.028 | 0.000 | 0.218 | 0.296 | 0.222 | 0.000 | 0.638 |
| **PBDE-207** | 0.050 | 0.031 | 0.000 | 0.189 | 0.024 | 0.013 | 0.000 | 0.118 | 0.071 | 0.044 | 0.000 | 0.189 |
| **PPBDE-209** | 0.520 | 0.346 | 0.000 | 1.706 | 0.230 | 0.125 | 0.000 | 1.283 | 0.757 | 0.614 | 0.000 | 1.706 |
| **∑PBDEs** | **7.217** | **4.060** | **0.000** | **30.760** | **0.941** | **0.843** | **0.000** | **2.080** | **12.353** | **9.552** | **0.000** | **30.760** |

| | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **2378-TCDD** | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 |
| **12378-PeCDD** | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 |
| **123478-HxCDD** | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 |
| **123678-HxCDD** | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 |
| **123789-HxCDD** | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 |
| **1234678-HpCDD** | 0.000 | 0.000 | 0.000 | 0.001 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.001 |
| **OCDD** | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 |
| **∑PCDDs** | **0.000** | **0.000** | **0.000** | **0.001** | **0.000** | **0.000** | **0.000** | **0.000** | **0.000** | **0.000** | **0.000** | **0.001** |
| **2378-TCDF** | 0.002 | 0.001 | 0.000 | 0.007 | 0.000 | 0.001 | 0.000 | 0.001 | 0.003 | 0.003 | 0.000 | 0.007 |
| **12378-PeCDF** | 0.002 | 0.001 | 0.000 | 0.022 | 0.000 | 0.000 | 0.000 | 0.001 | 0.004 | 0.002 | 0.000 | 0.022 |
| **23478-PeCDF** | 0.001 | 0.001 | 0.000 | 0.003 | 0.000 | 0.000 | 0.000 | 0.001 | 0.001 | 0.001 | 0.000 | 0.003 |
| **123478-HxCDF** | 0.000 | 0.000 | 0.000 | 0.001 | 0.000 | 0.000 | 0.000 | 0.000 | 0.001 | 0.001 | 0.000 | 0.001 |
| **123678-HxCDF** | 0.000 | 0.000 | 0.000 | 0.001 | 0.000 | 0.000 | 0.000 | 0.001 | 0.000 | 0.000 | 0.000 | 0.001 |
| **234678-HxCDF** | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 |
| **123789-HxCDF** | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 |
| **1234678-HpCDF** | 0.000 | 0.000 | 0.000 | 0.001 | 0.000 | 0.000 | 0.000 | 0.000 | 0.001 | 0.001 | 0.000 | 0.001 |
| **1234789-HpCDF** | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 |
| **OCDF** | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 |
| **∑PCDFs** | **0.006** | **0.004** | **0.000** | **0.023** | **0.002** | **0.002** | **0.000** | **0.003** | **0.009** | **0.008** | **0.001** | **0.023** |
| **∑PCDD/Fs** | **0.006** | **0.004** | **0.000** | **0.023** | **0.002** | **0.002** | **0.000** | **0.003** | **0.010** | **0.008** | **0.001** | **0.023** |

| | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **PeCB** | **0.112** | **0.103** | **0.040** | **0.222** | **0.061** | **0.056** | **0.040** | **0.085** | **0.153** | **0.148** | **0.095** | **0.222** |
| **HxCB** | **1.727** | **1.782** | **0.658** | **3.176** | **1.371** | **1.159** | **0.658** | **2.062** | **2.019** | **1.866** | **1.473** | **3.176** |
| **α-HCH** | 0.341 | 0.137 | 0.000 | 0.976 | 0.426 | 0.486 | 0.009 | 0.905 | 0.272 | 0.012 | 0.000 | 0.976 |
| **γ-HCH** | 0.428 | 0.293 | 0.037 | 1.217 | 0.508 | 0.605 | 0.199 | 0.897 | 0.362 | 0.292 | 0.037 | 1.217 |
| **β-HCH** | 0.250 | 0.186 | 0.000 | 0.741 | 0.032 | 0.025 | 0.000 | 0.113 | 0.429 | 0.498 | 0.174 | 0.741 |
| **∑HCHs** | **1.019** | **0.882** | **0.240** | **2.391** | **0.966** | **1.195** | **0.240** | **1.835** | **1.063** | **0.844** | **0.357** | **2.391** |
| **α-endosulfan** | 0.196 | 0.209 | 0.036 | 0.450 | 0.094 | 0.073 | 0.036 | 0.303 | 0.279 | 0.257 | 0.174 | 0.450 |
| **β-endosulfan** | 0.016 | 0.011 | 0.000 | 0.068 | 0.024 | 0.016 | 0.009 | 0.068 | 0.010 | 0.007 | 0.000 | 0.028 |
| **∑endosulfan** | **0.212** | **0.213** | **0.045** | **0.478** | **0.118** | **0.095** | **0.045** | **0.371** | **0.289** | **0.266** | **0.181** | **0.478** |
| **op'-DDE** | 0.031 | 0.012 | 0.004 | 0.142 | 0.020 | 0.011 | 0.007 | 0.048 | 0.040 | 0.012 | 0.004 | 0.142 |
| **pp'-DDE** | 136.306 | 125.129 | 5.572 | 349.647 | 22.061 | 15.086 | 5.572 | 55.615 | 229.778 | 250.366 | 71.737 | 349.647 |
| **op'-DDD** | 0.014 | 0.008 | 0.002 | 0.049 | 0.011 | 0.006 | 0.004 | 0.049 | 0.016 | 0.010 | 0.002 | 0.047 |
| **op'-DDT** | 0.087 | 0.042 | 0.011 | 0.359 | 0.042 | 0.032 | 0.011 | 0.094 | 0.124 | 0.060 | 0.015 | 0.359 |
| **pp'-DDD** | 0.718 | 0.203 | 0.011 | 3.055 | 0.031 | 0.031 | 0.011 | 0.056 | 1.280 | 0.861 | 0.140 | 3.055 |
| **pp'-DDT** | 1.781 | 0.204 | 0.000 | 12.111 | 0.135 | 0.084 | 0.000 | 0.540 | 3.127 | 1.951 | 0.094 | 12.111 |
| **∑DDTs** | **138.936** | **128.811** | **5.920** | **364.067** | **22.300** | **15.462** | **5.920** | **55.696** | **234.366** | **251.395** | **73.884** | **364.067** |
| **∑OCPs** | **142.007** | **131.370** | **7.222** | **368.628** | **24.816** | **18.778** | **7.222** | **58.030** | **237.890** | **254.509** | **76.569** | **368.628** |
| **∑POPs** | **437.399** | **469.207** | **17.407** | **975.441** | **104.149** | **32.058** | **17.407** | **317.546** | **710.058** | **712.233** | **345.913** | **975.441** |

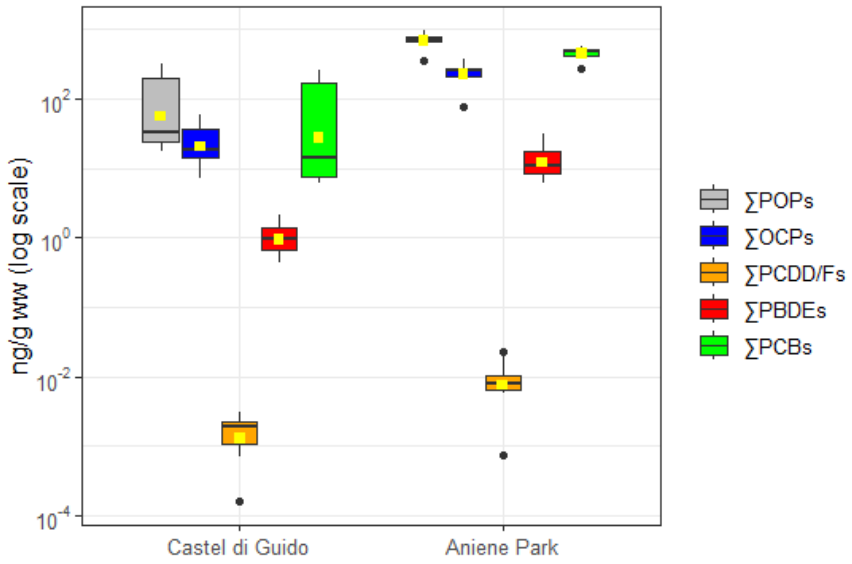


**Figure 3.15.** Boxplot of the target families in ng/g ww (log scale).

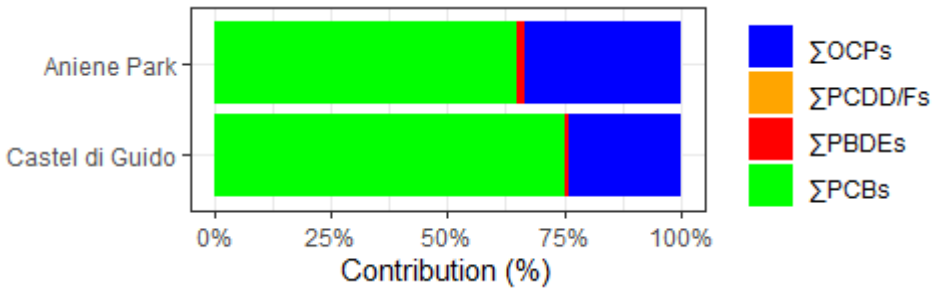


**Figure 3.16.** Target family's contribution.

Among the targeted OCPs, only ∑DDTs, ∑endosulfan, and PeCB show a significantly higher median value in Aniene Park compared to Castel di Guido, as supported by all three statistics ($p<0.05$; Figure 3.17). The presence of significantly elevated median values was observed for ∑HCHs based solely on the Wilcoxon rank-sum test. Notably, no significant differences were observed for HxCB. The predominant contribution to the ∑OCPs (median: 131 ng/g ww) profiles is primarily attributed to ∑DDTs (median: 129 ng/g ww), which in turn is mainly due to elevated levels of pp'-DDE (median: 125 ng/g ww) (Figure 3.17B; Figure 3.18A and 3.18B). Notably, HxCB and ∑HCHs depict a relatively high contribution, particularly in the Castell di Guido location (Figure 3.17.A). Interestingly, in this location, the α and γ isomers are notably similar, while the β isomer exhibits a considerably lower contribution (Figure 3.18C). Conversely, in Aniene Park, the contributions of the three isomers are

relatively similar, with a slightly higher presence of β-HCH (Figure 3.18C). The contribution of ∑endosulfan to the OCPs profile is minimal, primarily attributed to α-endosulfan (Figure 3.18D).

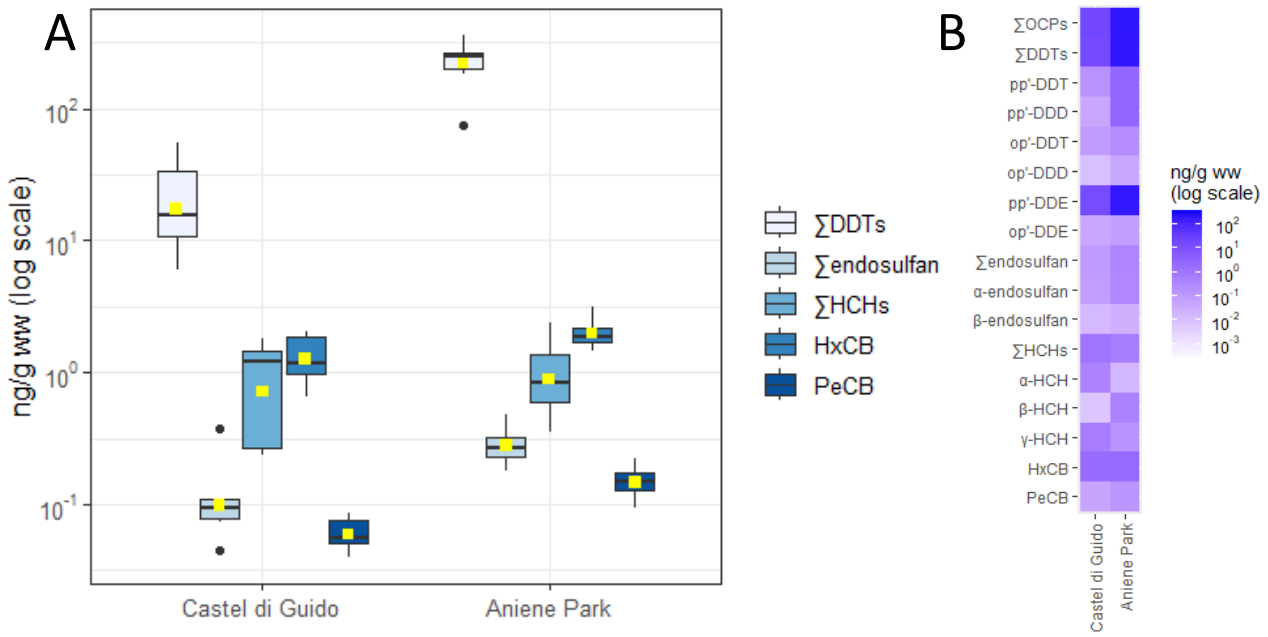


**Figure 3.17.** Boxplot (A) and heatmap (B) of the OCPs in ng/g ww (log scale).

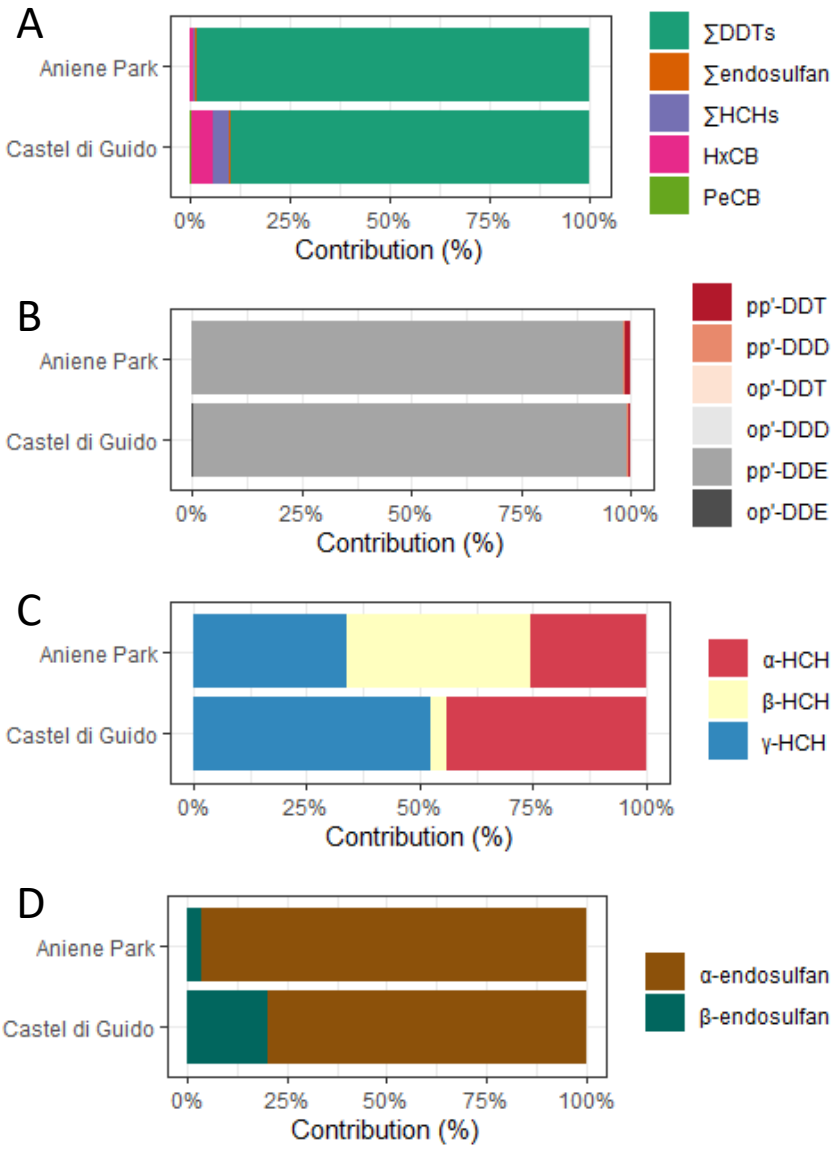


**Figure 3.18.** OCPs contribution (A), DDTs (B), HCHs (C) and endosulfan (D) isomers contribution.

Concerning non-pesticide POPs, ∑PCBs, ∑PBDEs, ∑PCDD/Fs, and ∑PCDFs show a significantly higher median value in Aniene Park compared to Castel di Guido, as supported by all three statistics ($p < 0.05$). Notably, no significant differences were observed for ∑PCDFs. ∑PCBs made the most substantial contribution, accounting for up to 75% of the POPs profile in Castell di Guido (Figure 3.16). The PCBs profile in both locations was predominantly composed of PCB-118, followed by PCB-153 (Figure 3.19A). In the case of PBDEs, PBDE-153 was the primary compound in both locations. However, PBDE-99 emerged as the subsequent prominent compound in Aniene Park, while in Castel di Guido, PBDE-209 held that position (Figure 3.19B). Regarding PCDD/Fs, the profiles varied between locations (Figure 3.19C). In Aniene Park, the dominant compounds were 12378-PeCDF,

followed by 2378-TCDF and 23478-PeCDF. In Castel di Guido, an equal dominance was observed for 2378-TCDF and 23478-PeCDF, followed by 12378-PeCDF.

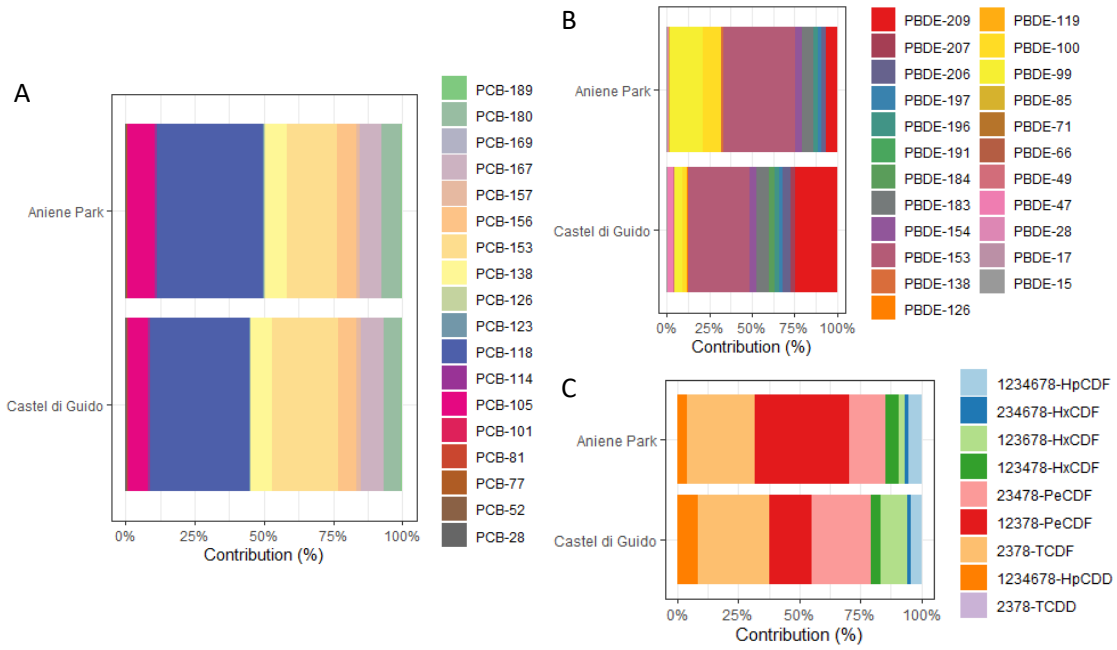


**Figure 3.19**. PCBs (A), PBDEs (B) and PCDD/F (C) contribution.

**Table 3.8.** Wilcoxon rank sum test (ng/g ww). P values <0.05

| Chemical | Statistic | p.value | method | alternative | effectsize | estimate | conf.level | conf.low | conf.high | conf.method | n.obs |
|---|---|---|---|---|---|---|---|---|---|---|---|
| **POP_sum** | **0** | **0.0002** | **Wilcoxon rank sum test** | **two.sided** | **r (rank biserial)** | **-1.000** | **0.950** | **-1.00** | **-1.00** | **normal** | **20** |
| **OCP_sum** | **0** | **0.0002** | **Wilcoxon rank sum test** | **two.sided** | **r (rank biserial)** | **-1.000** | **0.950** | **-1.00** | **-1.00** | **normal** | **20** |
| **DDT_sum** | **0** | **0.0002** | **Wilcoxon rank sum test** | **two.sided** | **r (rank biserial)** | **-1.000** | **0.950** | **-1.00** | **-1.00** | **normal** | **20** |
| **Endosulfan_sum** | **9** | **0.0024** | **Wilcoxon rank sum test** | **two.sided** | **r (rank biserial)** | **-0.818** | **0.950** | **-0.93** | **-0.56** | **normal** | **20** |
| HCH_sum | 42 | 0.5949 | Wilcoxon rank sum test | two.sided | r (rank biserial) | -0.152 | 0.950 | -0.59 | 0.35 | normal | 20 |
| **HCB_** | **19** | **0.0227** | **Wilcoxon rank sum test** | **two.sided** | **r (rank biserial)** | **-0.616** | **0.950** | **-0.85** | **-0.20** | **normal** | **20** |
| **PeCB** | **0** | **0.0002** | **Wilcoxon rank sum test** | **two.sided** | **r (rank biserial)** | **-1.000** | **0.950** | **-1.00** | **-1.00** | **normal** | **20** |
| **PCB_sum** | **0** | **0.0002** | **Wilcoxon rank sum test** | **two.sided** | **r (rank biserial)** | **-1.000** | **0.950** | **-1.00** | **-1.00** | **normal** | **20** |
| **PBDE_sum** | **0** | **0.0004** | **Wilcoxon rank sum test** | **two.sided** | **r (rank biserial)** | **-1.000** | **0.950** | **-1.00** | **-1.00** | **normal** | **18** |
| **PCDDF_sum** | **7** | **0.0014** | **Wilcoxon rank sum test** | **two.sided** | **r (rank biserial)** | **-0.859** | **0.950** | **-0.95** | **-0.65** | **normal** | **20** |
| **PCDF_sum** | **7** | **0.0026** | **Wilcoxon rank sum test** | **two.sided** | **r (rank biserial)** | **-0.841** | **0.950** | **-0.94** | **-0.59** | **normal** | **19** |
| PCDD_sum | 10 | 0.3502 | Wilcoxon rank sum test | two.sided | r (rank biserial) | -0.375 | 0.950 | -0.81 | 0.32 | normal | 12 |

**Table 3.9.** Yuen's test on trimmed means for independent samples (ng/g ww).

| Chemical | statistic | df.error | p.value | method | effectsize | estimate | conf.level | conf.low | conf.high | n.obs |
|---|---|---|---|---|---|---|---|---|---|---|
| **POP_sum** | **5.47** | **6.08** | **0.0015** | **Yuen's test on trimmed means for independent samples** | **Algina-Keselman-Penfield robust standardized difference** | **-1.537** | **0.950** | **-14.98** | **-0.93** | **20** |
| **OCP_sum** | **10.14** | **7.23** | **0.0000** | **Yuen's test on trimmed means for independent samples** | **Algina-Keselman-Penfield robust standardized difference** | **-2.984** | **0.950** | **-7.65** | **-1.92** | **20** |
| **DDT_sum** | **9.39** | **6.93** | **0.0000** | **Yuen's test on trimmed means for independent samples** | **Algina-Keselman-Penfield robust standardized difference** | **-2.732** | **0.950** | **-7.76** | **-1.96** | **20** |
| **Endosulfan_sum** | **10.08** | **11.95** | **0.0000** | **Yuen's test on trimmed means for independent samples** | **Algina-Keselman-Penfield robust standardized difference** | **-4.127** | **0.950** | **-7.62** | **-0.63** | **20** |
| HCH_sum | 0.34 | 10.25 | 0.7389 | Yuen's test on trimmed means for independent samples | Algina-Keselman-Penfield robust standardized difference | -0.114 | 0.950 | -0.76 | 0.57 | 20 |
| HCB | 2.28 | 7.79 | 0.0530 | Yuen's test on trimmed means for independent samples | Algina-Keselman-Penfield robust standardized difference | -0.685 | 0.950 | -2.54 | -0.14 | 20 |
| **PeCB** | **6.48** | **11.99** | **0.0000** | **Yuen's test on trimmed means for independent samples** | **Algina-Keselman-Penfield robust standardized difference** | **-2.526** | **0.950** | **-7.73** | **-1.63** | **20** |
| **PCB_sum** | **4.36** | **6.09** | **0.0046** | **Yuen's test on trimmed means for independent samples** | **Algina-Keselman-Penfield robust standardized difference** | **-1.226** | **0.950** | **-7.04** | **-0.88** | **20** |
| **PBDE_sum** | **9.14** | **9.96** | **0.0000** | **Yuen's test on trimmed means for independent samples** | **Algina-Keselman-Penfield robust standardized difference** | **-4.142** | **0.950** | **-10.43** | **-2.32** | **18** |
| **PCDDF_sum** | **6.74** | **8.86** | **0.0001** | **Yuen's test on trimmed means for independent samples** | **Algina-Keselman-Penfield robust standardized difference** | **-2.116** | **0.950** | **-10.56** | **-0.97** | **20** |
| **PCDF_sum** | **8.09** | **9.42** | **0.0000** | **Yuen's test on trimmed means for independent samples** | **Algina-Keselman-Penfield robust standardized difference** | **-3.092** | **0.950** | **-9.31** | **-1.85** | **19** |
| PCDD_sum | 1.25 | 7.46 | 0.2475 | Yuen's test on trimmed means for independent samples | Algina-Keselman-Penfield robust standardized difference | -0.567 | 0.950 | -7.54 | 0.52 | 12 |

**Table 3.10.** Asymptotic Two-Sample Brown-Mood Median Test (ng/g ww).

| Chemical | statistic | p.value | method |
|---|---|---|---|
| **POP_sum** | **-3.9428** | **0.0001** | **Asymptotic Two-Sample Brown-Mood Median Test** |
| **OCP_sum** | **-3.9428** | **0.0001** | **Asymptotic Two-Sample Brown-Mood Median Test** |
| **DDT_sum** | **-3.9428** | **0.0001** | **Asymptotic Two-Sample Brown-Mood Median Test** |
| **Endosulfan_sum** | **-3.0666** | **0.0022** | **Asymptotic Two-Sample Brown-Mood Median Test** |
| HCH_sum | 0.43809 | 0.6613 | Asymptotic Two-Sample Brown-Mood Median Test |
| HCB_ | -1.3143 | 0.1888 | Asymptotic Two-Sample Brown-Mood Median Test |
| **PeCB** | **-3.9428** | **0.0001** | **Asymptotic Two-Sample Brown-Mood Median Test** |
| **PCB_sum** | **-3.9428** | **0.0001** | **Asymptotic Two-Sample Brown-Mood Median Test** |
| **PBDE_sum** | **-3.6878** | **0.0002** | **Asymptotic Two-Sample Brown-Mood Median Test** |
| **PCDDF_sum** | **-3.9428** | **0.0001** | **Asymptotic Two-Sample Brown-Mood Median Test** |
| **PCDF_sum** | **-3.4325** | **0.0006** | **Asymptotic Two-Sample Brown-Mood Median Test** |
| PCDD_sum | -1.1726 | 0.2410 | Asymptotic Two-Sample Brown-Mood Median Test |

### 3.2. Conclusions of Common Kestrel ecotoxicology investigations

This study has gained new knowledges on the ecotoxicological status and physiological responses of Common Kestrel populations from areas with different anthropic pressures (natural, urban and rural) by applying a blood-based multi-biomarker approach combined with contaminant analysis.

We can draw the following specific conclusions:

- Sensitive genotoxicity (comet assay) and immunotoxicity (respiratory burst) biomarkers were developed for the first time on Common Kestrel in this thesis. The comet assay on the Kestrel was developed on fresh and frozen blood. Tests on fresh blood were carried out modifying the main parameters of the test: the dilution of samples in Phosphate Buffered Saline (PBS) and the timing of the electrophoretic run. The most reliable results were obtained with the following parameters: two dilutions (1:1000 and 1:4) and 10 minutes of electrophoresis time. Once the test on fresh blood was developed, the tests on frozen blood were conducted to determine the proper dilution in a cryopreservation medium (DMSO:RPMI, 20:80). The best concentration was 1:200. Subsequently, once determined the proper blood cryoconservation condition, the parameters developed for the fresh comet assay were tested and confirmed. Regarding the respiratory burst, we conducted three modifications to the reference method: incubation temperature of the plate, the stimulation of the cell membranes of leukocytes performing respiratory burst with PMA and the amount of blood sample. The best results were obtained with 40 °C incubation temperature and using the PMA. The tests developed have expanded the battery of biomarkers that can be used in this species, allowing the measurement of parameters fundamental for assessing the health status of the populations studied.
- We successfully validated the comet assay through *in vitro* studies on blood of Common Kestrel. Interesting information about the toxicological effects of two commercial pesticides were obtained. An herbicide (Round up) and a fungicide (Amistar®Xtra), that are widely used but rarely investigated, were tested. Firstly, we tested the viability of blood cells exposed to the two pesticides for different time exposure (1, 3, 6 and 24 h), we found that cells exposed to the fungicide (field use concentration 200 g/L) were vital until 6 hours, while those exposed to glyphosate (field use concentration 300 mL/L) just after 3 hours showed a viability of only 40%. Concentration of glyphosate were thus reduced, and a good viability was found at the

concentration of 4.68 mL/L. The comet assay was then applied to evaluate the potential genotoxic effects of the two compounds. Amistar®Xtra showed similar level of genotoxicity after 6 hours of exposure at field dose, while glyphosate showed a strong increase of DNA damage after 3 hours of exposure at below field dose. Glyphosate resulted to be strongly toxic to cells even at concentrations well below usage doses after 3 hours of exposure. As already underlined, glyphosate is the widest used herbicide, with controversial opinions about its toxicity potential, this first step of *in vitro* studies confirms the need to further *in vitro* and *in vivo* studies on the toxicity of this compound to non target species. The *in vitro* approach developed in this thesis have shown a good potential to test toxicity of commercial formulations of pesticides.

- In the field monitoring study, we found evidence for (i) higher immune activation (monocytes), alteration of oxidative status (lower GSH and GSH:GSSG) and interference with cholinesterase activity in urban and natural kestrels compared to rural kestrels, and (ii) higher DNA damage in rural kestrels compared to urban and natural kestrels. We also found that many of our markers were rather similar across the three study areas. These results suggest that kestrels probably face with environmental stressors that differ in typology or intensity across contrasting environments, so that concluding that cites are not adequate habitats for kestrels might be premature. Also, the between-year differences observed in various parameters suggest that conditions for each of the three habitats considered can vary across time, so that they need to be taken into account when comparing urban and non-urban populations. Our multi-marker approach has highlighted that kestrels are exposed to different environmental pressures, requiring adequate physiological responses. Indeed, we found important differences of contaminants concentrations among the studied environments. The urban area showed the highest concentration of non-pesticide POPs (∑PCBs, ∑PBDEs, ∑PCDD/Fs, and ∑PCDFs), OCPs (∑DDTs, ∑endosulfan, and PeCB) but also of Hg and Cu. Moreover, even the natural area has been found to be contaminated by POPs (HxCB and ∑HCHs) and heavy metals (Cd and Pb) that probably induced the recorded DNA damage and impairment of BChE activity. Future studies will be needed to better determine other drivers of this physiological differentiation among conspecific populations breeding in different habitats, and the potential consequences for population dynamics. This information is particularly relevant to guide exploitation of lands and urban planning in a more compatible way with the health of wildlife.

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https://www.r-project.org/

# CHAPTER 4

# ECOTOXICOLOGICAL INVESTIGATIONS ON GREAT TIT

# CHAPTER 4

## ECOTOXICOLOGICAL INVESTIGATIONS ON GREAT TIT

### 4.1. Introduction

#### 4.1.1. The great tit (*Parus major* Linnaeus, 1758)

The great tit, *Parus major* (Linnaeus, 1758) (Figure 4.1.), is a species belonging to the Paridae family. The world population of great tit is between 430 to 709 million mature individuals. It is a species with little migratory tendency and frequents various environments, from agro-forest areas to urban green spaces. The great tit has a wide distribution; in fact, it can be found throughout Europe, North Africa, the Middle East, and part of Central Asia and can survive up until an upper elevation limit of 4,420 meters above sea level (BirdLife, 2016).

**Figure 4.1.** The great tit (*Parus major*, Linnaeus, 1758) (Photo: lipu.it).

Its length may vary between a range of 12.5 to 15 cm, while its wingspan is usually around 23-26 cm and weight can differ from 14 to 22 grams (Mullarney et al., 1999). *Parus major* is defined by its black crown and neck, large white cheek patches and a black band running down the centre of a bright yellow breast (Beaman & Madge, 2010). Unlike other members of the Paridae family, the great tit presents a sexual dimorphism, which consists in the different size of the black breast stripe (Norris, 1990).The size, as described by Kolliker et al. (1999), is a trait that does not vary as much between sexes. *Parus major* is mainly an insectivorous species with a particular preference for lepidopteran larvae (caterpillars) especially for what concerns nestling food (Sinkovics et al., 2021); if insects are scarce, berries and seeds may be added to its diet (Groffen et al., 2020). It is a cavity nester that easily tends to breed in artificial nests, such as nest boxes, as these provide a valuable nesting resource (Lee et al., 2023). The breeding season in Mediterranean regions takes place from

March to June (Solís et al., 2023a). The clutch size can vary from 6 to 11 eggs, and the incubation period is estimated to be 13 days (Lee et al., 2023). The great tit is a monogamous species and both parents feed their young at the nest (Kolliker, 1999). Nestlings' fledging date occurs on day 17 to 20 of age (Naef-Daenzer & Grüebler, 2008), great tits life expectancy is of 4.3 years (BirdLife, 2016).

### 4.1.2. *Parus major* in ecotoxicological studies

We selected the great tit as a study species, because it is a cavity nesting species and it can nest both in natural and artificial cavities, such as man-made nest boxes. Given the propensity of great tit to breed in artificial nests, it is easy to catch, handle and sample and, as regards nestlings, the use of traps is not necessary. The great tit is a widespread species spreading across forests, agricultural and urban environments; thus, this makes possible to study the impact of environments with different anthropogenic pressures on the species. Its residentiality and the limited home range are other aspects that permitted to choose this species for this study. These characteristics allow to obtain a good "picture" of the anthropogenic pressures on a given territory, without variables deriving from migration; thus great tits can reflect a potential local contamination. The great tit has a high position in the food chain and its diet consists mainly of caterpillars during the breeding season and in winter, when insects are scarce, and it can consume seeds, buds, nuts and berries. The diet habits are particularly relevant because determine a possible exposure of the great tits to different substances of anthropogenic origin. For instance, being on trees and tumbling in the ground, in search of food, great tits could come into contact with substances adsorbed to the leaves or could be exposed to plant protection products when they consume plant materials.

The available literature on ecology, behavior and reproduction are extensive and this provides an excellent background knowledge to address the studies (Song et al., 2020; Hollander et al., 2008; Kvist et al., 2003; Björklund et al., 2009; Grabowska-Zhang et al., 2012; Ouyang et al., 2013).

Considering these aspects, the great tit can be deemed a good model species in ecotoxicological field studies.

### 4.1.3. Threats to great tit

At a global level, the IUCN red list (International Union for Conservation of Nature) classifies *Parus major* as least concern (LC) and the main threat that causes an impact on this species is the climate change. Nonetheless, anthropogenic environmental alterations, such as a rapid increasing urbanisation are also to be counted as threats to the species (Sinkovics et al., 2021). In the last seventy years, the human population has grown quickly (www.un.org) and there has been a

corresponding increase in urbanisation. This trend can lead to a reduction of habitat through its fragmentation (Corsini et al., 2021). Insectivorous birds, such as *Parus major*, provide a good example of a wildlife population impacted by urbanisation; in fact, it's demonstrated that there is a lower reproductive success in urban specimens compared to the ones that live in more natural habitats and this can be due to a low availability of high-quality food for offspring (Sinkovics et al., 2021). The clearest pattern of modification of the climate is seen in the advancement of the breeding period (Solís et al., 2023b). The increasing spring temperatures due to climate change, can cause modifications in organisms reproductive phenology. Insect eggs hatch earlier, influencing the reproductive timing of secondary consumers such as insectivorous birds (including *P. major*), which must synchronize their reproductive peak with the slightly advanced food availability. This temporal shortening in food availability has a strongly negative impact on the reproductive success of great tits, leading to a decreased likelihood of a second deposition and generally reducing the temporal span of the breeding season (Andreasson et al., 2023). Moreover, the rise of the global population increases food demand; therefore, natural environments are not only destroyed due to urbanisation, but also due to the increase in land used for agricultural purposes (www.fao.org). Pesticides and chemical treatments in general that are used in agriculture cause an impact on great tit; a significant reduction in breeding pair density and, to a lesser extent, a reduced nesting success during incubation period can occur. The use of insecticides also leads directly to a lower food availability which then can be connected to a high rate of aborted nests during incubation (Bouvier et al., 2005).

### 4.1.4. State of the art on great tit ecotoxicological studies

The great tit is considered an excellent model species for this reason it has been used in monitoring studies. To date, several studies investigated the levels of various contaminants such as heavy metals, PCBs, OCPs and more in general POPs on great tit.

For example, when it comes to the evaluation of heavy metal levels in feathers of *Parus major* several studies were published over the past years. Markowski and collaborators (2014) examined heavy metal concentrations in feathers of nestling great tits and blue tits collected in two different sites, an urban parkland and a deciduous forest, in central Poland. Lead concentration, in great tit feathers was 4.41±0.84 μg/g and 4.14±0.84 μg/g in parkland and forest sites, respectively. Concentration of cadmium was double in forest site (0.86±0.30 μg/g) respect to parkland site (0.43±0.14 μg/g), whereas zinc levels very similar in the two areas (148.20±10.13 μg/g in parkland site and 153.49±5.24 in forest site). In another more recent study (Chatelain et al., 2021), heavy

metal (Cu, Zn, Pb, As and Hg) levels were analyzed in nestling feathers of great tits from two parks of Warsaw city and a rural forest. Chatelain and collaborators (2021) identified the heavy metals as responsible for the strongest effects on reproductive success. The associations between heavy metal exposure and reproductive outputs were independent of the habitat type. Authors found that in feathers Cu had a concentration of 7.5±0.2, Zn of 214.7±3.5, Pb of 0.9±0.4, As of 2.1±0.3 and Hg of 2.0±0.3. Persistent organic pollutants, such as polychlorinated biphenyls (PCBs), organochlorine pesticides (OCPs) and polycyclic aromatic hydrocarbons (PAHs), have been evaluated massively in the past. High concentrations of these pollutants can be found in the environment and pose a threat to many wildlife populations, including birds, as these contaminants can accumulate in living organisms and cause negative effects. POPs (PCBs, DDTs, HCB and PBBs) were evaluated in both fat and feathers of the great tit to investigate possible temporal and spatial differences (Dauwe et al., 2005). PCBs, OCPs and PBDEs concentrations were also evaluated in great tit eggs sampled in rural, industrial and agricultural sites in Belgium. Levels of ∑PCBs and ∑PBDEs were significantly higher in the industrial areas compared to the other locations and their levels reached up to 6050 and 79 ng/g lw, respectively. On the contrary, significantly higher levels of OCPs (∑OPCs up to 2683 ng/g lw) were detected in the rural sampling locations (Van den Steen et al., 2008). Another study of Van den Steen and collaborators (2006) was conducted on great tit eggs to investigate the concentration variation of PCBs, PBDEs and OCPs contaminants within and among clutches and to determine whether egg laying order affects the concentration of these contaminants. PCBs were the major organohalogenated compounds (mean 4778 ng/g lw), while PBDEs (mean 204 ng/g lw) and DDTs (mean 601 ng/g lw) were found at much lower concentrations. They found no difference in PCBs, PBDEs and OCPs levels within-clutch and laying order did not affect the concentrations these compounds. Recently great tit eggs have been also used to evaluate the presence of emerging contaminants in a given environment. Among emerging contaminants per- and polyfluoroalkyl substances (PFAS) are globally distributed and have raised increasing concerns in recent decades due to their persistence in the environment and toxicity to human and wildlife (Lopez-Antia et al., 2013). Although some PFAS are regulated, the presence of their precursors and alternatives in the environment requires frequent monitoring. Bird eggs have been proven to be a good monitoring tool for assessing the presence and exposure to PFAS (Morganti et al., 2021). Groffen et al. (2017) investigated the levels of PFAAs (PFSAs and PFCAs) in great tits eggs collected in a fluorochemical plant and in three other areas in Belgium, representing a gradient in distance from the pollution source. PFSA concentrations detected in the fluorochemical plant were 10380 ng/g, 99.3 ng/g and

47.7 ng/g for PFOS, PFHxS and PFDS respectively. Also, the median concentration of PFOA (19.8 ng/g) was among the highest ever reported in bird eggs. Another study conducted near a fluoro-chemical plant in Belgium on eggs of great tit showed considerable concentration of PFOS (19 -5635 ng/g ww) (Lopez-Antia et al., 2017). High PFAS levels can affect birds' reproduction. Groffen and collaborators (2019b) found a correlation between high concentrations of PFDA and a reduced hatching success, advanced egg laying, and a reduction of breeding success in great tit eggs. Interestly, Lasters et al. (2019) found that PFAA concentrations is associated with laying sequence of great tit eggs.

To date, several studies evaluated the oxidative status and potential oxidative stress effects in *Parus major* from urban and industrial environments (Isaksson et al., 2009; Geens et al., 2009; Koivula et al., 2011; Rainio et al, 2015; Buytaert et al., 2023). However, no studies evaluated the porphyrin levels and applied the comet assay and ENA assay on great tit. A test similar to the ENA assay, or the micronucleus test, was instead used on the species we studied (Drahulian et al., 2018). Moreover, also at immunological level we found some shortcomings. For instance, the respiratory burst process (very important because it is linked to other important physiological parameters of an organism) was evaluated recently in adult free-living great tits to investigate potential correlation between this process and age (Těšický et al., 2021). This test was applied in ecotoxicology field for the first time in this work of thesis. On the contrary, some work on other biomarkers of immunotoxicity applied in this study was published, but always in a reduced number (Vermeulen et al., 2015; Bauerová et al., 2017; Bauerová et al., 2020). The BChE activity was assessed in some works conducted on adult and nestling great tit living in woodlands, suburban or industrial environments (Norte et al., 2008a, Norte et al., 2008b, Norte et al., 2009; Norte et al., 2010), while CaE activity was less investigated (Cordi et al., 1997). Furthermore, based on our knowledge, most of the studies evaluated one or a few endpoints without applying a muti-biomarkers approach.

The effects of contaminants can occur at the behavioural level too, with a reduction in the singing repertoire of male passerine birds from areas polluted with heavy metals (Gorissen et al., 2005), as well as cause the impairment of reproductive success in birds exposed to contaminants of urban and industrial emissions (Sanderfoot & Hollwayet, 2017).

Insectivorous birds, such as *Parus major*, provide a good example of a wildlife population impacted by urbanisation; in fact, it's demonstrated that there is a lower reproductive success in urban specimens compared to the ones that live in more natural habitats and this can be due to a low availability of high-quality food for offspring (Sinkovics et al., 2021). The clearest pattern of

modification of the climate is seen in the advancement of the breeding period (Solís et al., 2023b). The increasing spring temperatures due to climate change, can cause modifications in organisms reproductive phenology. This temporal shortening in food availability has a strong negative impact on the reproductive success of great tits, leading to a decreased likelihood of a second deposition and generally reducing the temporal span of the breeding season (Andreasson et al., 2023). The use of insecticides also leads directly to a lower food availability which then can be connected to a high rate of aborted nests during incubation (Bouvier et al., 2005).

The available literature on ecology, behavior and reproduction is extensive and provides an excellent background knowledge to address the studies (Song et al., 2020; Hollander et al., 2008; Kvist et al., 2003; Bjorklund et al., 2009; Grabowska-Zhang et al., 2012; Ouyang et al., 2013).

## 4.2. Aim

Climate change, urbanization, and agricultural activities lead to habitat modification, food availability and quality reduction, and increased emissions of compounds harmful to birds. Indeed, many bird species are listed in the IUCN Red List of Threatened Species. Since 2016, great tit (*Parus major*) has been classified as Least Concern, and the global population is considered to be increasing. Great tit can be considered a suitable model species since it is a ubiquitous species, mainly insectivorous during the breeding season, therefore high in the food chain, forages in small home ranges reflecting local contamination, and is easily monitored through nest boxes that the animals use for the laying. The species is affected by several anthropogenic pressures such as higher spring temperatures due to climate change, which results in a mismatch between food availability and offspring requirements and may lead to lowered reproductive success. Another pressure is pesticide use and the emission of other contaminants in urban and industrial environments.

To date, although great tit is often used as a model species, there are still significant gaps in studies conducted on this species. There is a preponderance of studies carried out in urban and industrial environments compared to agricultural ones. Moreover, these studies mainly evaluate only one or very few endpoints, ignoring that environmental stressors affect animals in different ways and, therefore, different toxicological and biological responses should be taken into account to have a real vision of, in parallel, species and environment's state. Thus, there is a need for more studies that apply a multi-biomarker approach and studies that embrace a holistic approach.

As already mentioned, since great tit is exposed to different environmental pressures arising from human activities, a more structured approach is increasingly needed. A multi-biomarker and

holistic/complementary approach that evaluates sub-lethal effects at different levels of biological organization, from sub- and individual level up to population level, combined with chemical analysis data, could be a valid solution for ecotoxicological studies.

In this contest, this part of the work of thesis aims to evaluate the ecotoxicological status of great tit populations from areas with different anthropic impacts using a multi-biomarker and complementary approach based on contaminant analysis, biomarker responses, behavioural traits and breeding performances.

The area of study will be the city of Padua and neighbouring areas. The Po Valley is the most economically prosperous Italian territory, with extended areas used for industry and agriculture, and consequently also one of the most contaminated regions. This territory was interested by a serious surface and groundwaters contamination by perfluoroalkyl carboxylic acids (PFAA) discharges from a fluorochemical plant. Our study areas are not included in the strongly PFAS contaminated sites, however some of our study areas are not far from them. Moreover, the Veneto Region dominate the European ranking for air pollution and among them there is the province of Padua.

To achieve the main aim of the study, specific objectives will be:

- To evaluate the presence of different classes of contaminants in non-lethal matrices (feathers and eggs) of great tit from wooded, urban and agricultural areas of Padua city and its surroundings. Specifically, to evaluate the levels of heavy metals (Cd, Pb, Hg, Cu) in feathers; to evaluate the levels of OCPs (DDTs, endosulfuran, HCHs, PeCB and HxCB) and PFAS in abandoned and unhatched eggs collected during nests monitoring;
- To evaluate potential differences in the concentrations of each class of contaminants among the different study areas;
- To apply a set of biomarkers on blood of great tit nestlings sampled in the different study areas to assess different toxicological responses at molecular, biochemical and cellular level. Specifically, oxidative status (Total Antioxidant Status, TAS), porphyrin levels, immunotoxicity (complement system activity, respiratory burst and differential White Blood Cells (WBCs) count), genotoxicity (comet and Erythrocytes Nuclear Abnormalities (ENA) assays), and neurotoxicity effects (butyrylcholinesterase and carboxylesterase (BChE and CaE) activities) of great tit nestlings will be evaluated. Effects of sampling year, sampling day and body mass (a proxy of health state of animals) for each biomarker will be studied;

- To evaluate differences in toxicological responses among populations from wooded, urban and agricultural study areas;
- To integrate the overall biomarker responses;
- To evaluate behavioural traits of great tit nestlings from the different study areas in order to assess potential alterations at the individual level. Specifically, the emotional and agitation state (breath rate, agitation test and tonic immobility) of great tit nestlings will be investigated. Moreover, the effects of sampling year, sampling day and body mass on the responses of each behavioural trait will be studied. Finally, potential differences in behaviour and personality among great tit nestlings from the study areas will be explored;
- To monitor the reproductive period of great tits from eggs laying to fledging in order to quantify reproductive success and potential effects at population level. Specifically, laying date, clutch size will be determined and hatching and fledging success of great tit populations will be quantified. The potential effects of sampling year on reproductive performances will also be evaluated;
- To evaluate potential differences in the different aspects of the reproductive success among the great tit populations from wooded, urban and agricultural areas. The possible effects of the contamination on these aspects will be investigated;
- To integrate the biomarkers responses with contaminant analysis data and information on behavioural traits and reproduction success parameters for a more complete evaluation of the toxicological status of the species, from molecular to population level;
- To evaluate how environments can influence the health status of great tit population studied and to understand if great tits adapt their physiology and behaviour to counter challenges from environmental stressors.

## 4.3. Heavy metals, POPs and PFAS contaminants on feathers and eggs of great tit

### 4.3.1. Material and methods

#### 4.3.1.1. Study areas

This study was conducted in the four different areas within the province of Padua (Veneto Region, Italy). Specifically, the sampling sites included the city of Padua, Euganean Hills Regional Park, Cà di Mezzo Oasis, and the locality of Vaccarino. These areas exhibited varying degrees of anthropogenic influence and can be categorized as urban, wooded, and two agricultural areas, respectively. The two agricultural areas Cà di Mezzo Oasis and Vaccarino were distinguished in agricultural 1 (hereinafter “agri 1”) and agricultural 2 (hereinafter “agri 2”), respectively. Information on the features of the study areas have been described in detail below (see section 4.4.1.1.).

#### 4.3.1.2. Feathers and eggs collection

The sampling campaigns were conducted between the middle of March to the end of May in 2021 and 2022.

The feathers were plucked from the breast of 159 nestlings (42 nests) with 14-15 days of age after carrying out the blood sampling. Feathers were stored at room temperature in paper bags until heavy metal analysis.

A total of 47 unhatched or abandoned eggs were collected from 18 nests during their routine inspection. Eggs were stored until further laboratory processing and analysis of persistent organic pollutants (POPs) and per- and polyfluoroalkyl substances (PFAS). Due to the small amount of egg content, we could not use the same egg for both contaminants analysis (POPs and PFAS), but eggs from the same nest were employed for both contaminants. Specifically, 20 eggs were used for POPs detection while 27 were applied to investigate the presence of PFAS.

#### 4.3.1.3. Analytical methods

**Analysis of heavy metals in feathers**

Feathers from pullets belonging to the same nest were merged (pool) and washed several times with distilled water to get rid of potential superficial contamination (Figure 4.2). Then the feathers were left to dry under hood, covered with clean filter paper. At the time of analysis, they were homogenized by shredding. Approximately 0.1 g of the homogenized samples were placed in Teflon containers and 2 ml of nitric acid ($HNO_3$) and 0.5 ml of hydrogen peroxide ($H_2O_2$) was added. The

containers were closed in a stain steel block and heated at temperature of 160 °C for 10 hours to complete the disintegration of organic matter. The obtained solutions were then transferred into polyethylene test tubes and made up to volume of 10 ml with ultrapure water and used for the analytical determination of heavy metals with atomic absorption spectrometers. In detail, Hg analysis was performed using cold vapor technique with a spectrometer Perkin Elmer Mod. FIMS 400 (Flow Injection Mercury System) and Cd, Cu and Pb were performed using Graphite Furnace technique with a spectrometer Analytik Jena Mod. ContrAA 700. In order to verify any contamination of reagents and the accuracy of the results, in each analysis batch a blank solution and standard references materials were always run. To obtain the calibration curve, internal additions method was used: standard solutions at increasing known concentrations of the analysed element were added to a sample replicate. The result is expressed as mg/kg dry weight (d.w.).

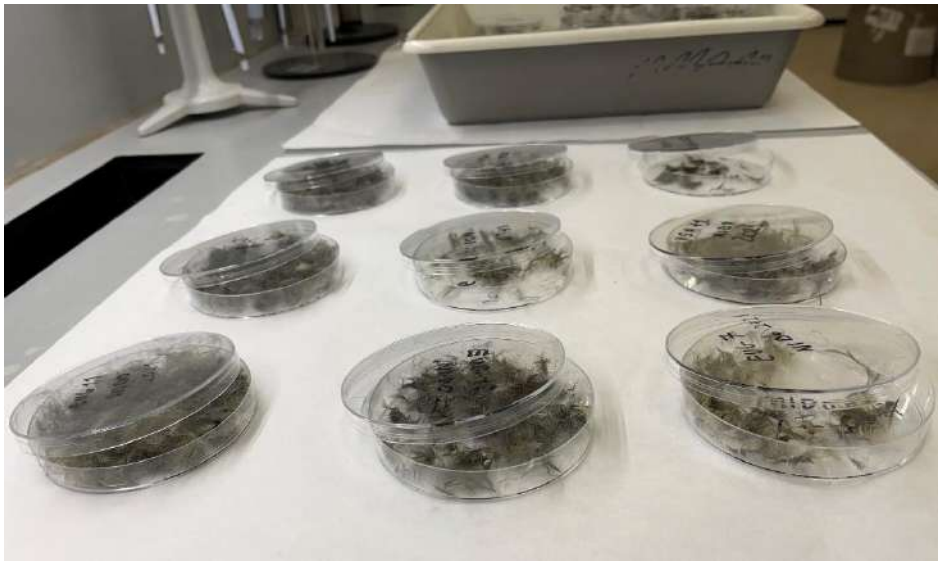

**Figure 4.2.** Washed feathers of great tit nestlings.

**Analysis of POPs in eggs**

*Chemical Treatment and Analysis of Samples*

The eggs were stored at -80ºC until processing. Once thawed, they were homogenized and lyophilized to remove their aqueous content. Throughout all stages related to the analytical treatment of the egg samples, the materials used (glass, plastic, or metal) were thoroughly cleaned before use. They were soaked overnight in Nuclean soap (National Diagnostics, Georgia, USA, specialized for the removal of isotopically labeled compounds), then rinsed, dried with acetone, and

washed thrice with each of the following solvents in decreasing order of polarity: acetone, dichloromethane, and n-hexane.

*Extraction*

For extracting the target analytes, a protocol employing ultrasonication was followed. Approximately 0.2 g of lyophilized egg material was homogenized with 1.5 g of anhydrous sodium sulfate in a mortar and introduced into a 25 mL cylindrical flask. Then, they were fortified with a known amount of isotopically labeled compounds. Specifically 20 µL of a solution containing 18 congeners of 13C12-PCB, 10 µL of a solution containing 26 congeners of 13C12-PBDEs, 10 µL of a solution containing 17 congeners of 13C12-PCDD/F, and 15 µL of 13C-organochlorine pesticides were added (namely, 13C6-PeCB, 13C6-HxCB, 13C6-α-HCH, 13C6-ɣ-HCH, 13C10-p,p-DDE, 13C10-o,p'-DDT, 13C10- p,p'-DDT, 13C9-α-endosulfan and 13C9-β-endosulfan).

After fortification, 10 mL of a cyclohexane:acetone mixture (3:1, v/v) was added. The flask containing the solvent and sample underwent sonication in an ultrasonic bath (J.P. Selecta, Barcelona, Spain) at room temperature for 15 minutes. This sonication process was repeated three times, changing the solvent between each cycle, yielding a final volume of approximately 30 mL in a 200 mL glass cell of a TurboVap® evaporation system (Zynmarck Inc., Hopkinton, Massachusetts, USA). Subsequently, the extract was concentrated to a final volume of around 1 mL using nitrogen at 0.8 bars and a temperature of 40°C in the TurboVap® system.

The final volume was transferred to a 15 mL Falcon tube along with a wash of the TurboVap® cell performed with cyclohexane:ethyl acetate (1:1, v/v) and brought up to 5 mL using the same solvent mixture. Samples were centrifuged at 2000 rpm for 5 min at room temperature, and the supernatant was transferred to a 15 mL cylindrical glass cell.

*Purification*

The purification stage aimed to separate lipids and/or impurities co-extracted with the target analytes in the previous step. To ensure maximal removal of undesired compounds, two distinct purification procedures were conducted for each sample.

The first utilized the GPCuno system (LCTech GmbH, Dorfen, Germany), an automatic gel permeation chromatography system (GPC). The elution of the sample employed a cyclohexane:ethyl acetate solvent mixture (1:1, v/v), injecting the 5 ml of the extract obtained in the previous step into the equipment using a glass syringe. The process lasted 55 min, collecting three different fractions. Only the first two fractions were collected in TurboVap® cells. Fraction I (110mL) contained lipids

and larger molecules, fraction II (110 ml) contained the target analytes, and fraction III (40 mL) corresponded to column cleaning and was discarded. Both fractions (I and II) were concentrated to 1 mL using the TurboVap® system.

The milliliter from fraction II was transferred to a beaker along with two washes of the cell using n-hexane:dichloromethane (9:1, v/v) and brought up to a volume of 4 mL using the same mixture, which was then divided into two aliquots.

One mL was collected in a vial (previously heated at 400°C to ensure the absence of any target analytes), used for pesticide content analysis. The other 3 ml were collected in a TurboVap® vessel and concentrated to 1 mL.

This resulting milliliter from the second aliquot underwent re-purification by adsorption chromatography using a 10 mL glass column, packed with 1.5 g of 44% weight-modified acidic silica with sulfuric acid and an approximate amount of granular anhydrous sulfate both below and above the silica. After conditioning the column with 8 mL of n-hexane:dichloromethane (9:1, v/v), the sample was loaded and eluted with 10 mL of the same solvent mixture. The purified eluate was collected in a TurboVap® cell, concentrated to 1 mL, and then transferred to a vial.

As a result, each sample yielded two vials vials, each containing 1 mL. One was used to measure the pesticide content, and the other for PCB, PBDE, and PCDD/F content analysis.

Additionally, fraction I containing lipid content was collected in a pre-weighed topaz vial using a precision balance.

*Concentration*

The contents of the vials were evaporated to dryness using nitrogen at 40°C with a Pasvial apparatus (Model V3, HiTC. S.A., Spain). Once dried, the vials were reconstituted with a mixture of 13C isotopically labeled standards as injection standards.

For the aliquot used to analyze pesticides, a volume made up of 10 µL of PCB IS and 10 µL of n-nonane was used. For the aliquot used to analyze the rest of POPs, 10 µL of PCB IS, 5 µL of PBDE IS, and 5 µL of PCDD/F IS were added. After reconstitution, the vials were vortexed and stored in a freezer until instrumental analysis.

Simultaneously, the vial with fraction I was concentrated using Pasvial until complete solvent evaporation. It was then placed in an oven at 105°C for 30 min. After cooling to room temperature overnight, the vial was reweighed on a precision balance to determine the weight of the extracted organic matter, representing the lipid content of the samples.

*Instrumental Analysis*

Identification and quantification of 8 mono-ortho substituted PCBs (105, 114, 118, 123, 156, 157, 167, and 189), 4 non-ortho substituted PCBs (77, 81, 126, and 169), 6 indicator PCBs (28, 52, 101, 138, 153, 180), 17 congeners 2,3,7,8-substituted of PCDD/Fs, and 26 PBDE congeners were performed using gas chromatography (GC) coupled with high-resolution mass spectrometry (HRMS) on a Trace GC Ultra chromatograph (Thermo Fisher Scientific, Milan, Italy) connected to a DFS magnetic sector mass spectrometer (Thermo Fisher Scientific, Bremen, Germany). 1 µL of each extract was injected in splitless mode at 260 °C using helium as the carrier gas. PBDE separation used a Rxi-5Sil ms column (dimensions 15m x 0.25 mm x 0.10 µm, Restek, USA), while PCB and PCDD/F separation used a DB-5ms column (dimensions 60m x 0.25 mm x 0.25 µm Agilent J&W, USA). The mass spectrometer used an electron impact source (at 45 eV) for analyte ionization, operated in selected ion monitoring (SIM) mode with two major ions for each analyte and a resolution of 10000 at 10% valley height. Analyte quantification was carried out using the isotopic dilution method. Detailed instrumental parameters can be found in previous works (Bartalini et al., 2019; Morales et al., 2022).

Identification and quantification of organochlorine pesticides (PeCB, HCB, p,p'-DDT, o,p'-DDT, p,p'-DDE, o,p'-DDE, p,p'-DDD, o,p'-DDD, α-HCH, β-HCH, γ-HCH, α-endosulfan, and β-endosulfan) were carried out using GC coupled to a low-resolution tandem mass spectrometer with a triple quadrupole analyzer (GC-QqQ-MS/MS). Helium was used as carrier gas. The gas chromatograph used was a 7890B (Agilent, Palo Alto, CA, USA) equipped with a programmable temperature vaporization injector (PTV) operating in splitless mode and a DB35-ms column (dimensions 30 m x 0.25mm x 0.25 µm, Agilent J&W, USA). The mass spectrometer was a 7010B (Agilent, Palo Alto, CA, USA) equipped with a high-efficiency 70eV electron impact ionization source. A source temperature of 260°C and quadrupole temperatures of 150°C were used. A volume of 1 µL was injected, and the multiple reaction monitoring (MRM) mode was employed, specifically monitoring two transitions for each analyte.

**Analysis of PFAS in eggs**

*Standards and Reagents*

LC–MS grade acetonitrile, LC–MS grade methanol, concentrated formic acid, ammonium hydroxide (25% in water) and ammonium acetate were purchased from Sigma-Aldrich (Missouri, US). All other reagents were at least analytical reagent grade. Clean water (DQ, 18.2 MΩcm resistivity) was

produced by a Millipore Direct-QUV water purification system (Millipore, Massachusetts, US). Certified mixture solution containing $C_6$-$C_{14}$ perfluoroalkyl carboxylates (PFCA), $C_4$-$C_6$-$C_8$-$C_{10}$ perfluoroalkyl sulfonates (PFSA), perfluorooctane sulfonamide (FOSA) and 6:2, 8:2 fluorotelomer sulfonates (FTS), ADONA (CAS 958445-44-8) and HFPO-DA (CAS 62037-80-3; also known as GenX) were purchased by (Wellington Laboratories, Ontario, Canada). Analytical standard of perfluoro([5-methoxy-1,3-dioxolan-4-yl]oxy)acetic acid (C6O4) (P5MeODIOXOAc, CAS Number 1190931-41-9) was supplied by Ultra Scientific (Italy) on the behalf of Solvay Specialty Polymers (Bollate, Italy). Commercial certified native solutions and methanolic stock solutions of C6O4 were diluted in methanol to obtain mixed native standard solutions at different concentration. Mass-labelled MPFAC-MXA solution (Wellington Laboratories) and mass-labelled d-N-EtFOSA-M solution (Wellington Laboratories.) were diluted in methanol (40 µg/L) for the preparation of the stable isotope labelled solution used as internal standard mixture (SIL-IS). Details on the analyte names and abbreviations are reported in Table 4.1.

*Sample extraction and analysis*

First the eggs were cleaned externally, measured and weighed. Eggs were then opened and the fresh weight of their content was recorded (about 0.6 -1 wet wt). Afterward the egg content was freeze-dried at -20°C and again weighed to record dry weight.

Great tit eggs were extracted according to Mazzoni et al. (2016). The dry content of the egg (0.1-0.2 g dry wt) were weighted into a PP centrifuge tube and spiked with 100 µl of 40 µg/L of SIL-IS solution. 5 mL of water and acetonitrile solution (10:90 v/v) were added to the solid sample. 70 µL of formic acid were added to the extraction mixture and vigorously shaken. Subsequently the tube was immersed in an ultrasonic bath for 15 min and then centrifuged for 12 min at 8,000 rpm at 10 °C. The extraction was repeated twice and the combined supernatants were transferred in a new 50 mL PP tube where 2 g $MgSO_4$ and 0.5 g NaCl were added. The tube was immediately shaken to prevent coagulation of $MgSO_4$, centrifuged and stored at −4 ◦C for one night.

The surnatant of all samples was reduced to 1 mL under a gentle nitrogen stream. To remove phospholipids, volume reduced extracts (1 mL) of egg samples were acidified (50 µL formic acid) and filtered through HybridSPE®Phospholipid Ultra cartridges (30 mg, 1 mL SPETubes, Sigma Aldrich), previously cleaned with 3 mL of acetonitrile and 50 µL of formic acid.

*Instrumental Analysis*

PFAS in the final extract were determined by liquid chromatography tandem mass spectrometry (UHPLC-MS/MS) coupled to a turbulent flow chromatography (TFC) for the online purification of the extracts (Mazzoni et al., 2016). Legacy and novel PFAS were determined using a modified Thermo EQuan system, which consists of a CTC PAL autosampler equipped with four 6-way VICI valves, two Thermo Scientific Accela LC pumps (600 and 1200) equipped with serially connected TFC (Thermo Fluoro XL, 50 × 0.5 mm and Thermo CycloneTM,50 × 0.5 mm) and analytical (Waters Acquity UPLC BEH C18, 1.7 µm 2.1 x 50 mm) columns. The TFC system was connected to a triple quadrupole mass spectrometer (Thermo Scientific TSQ Quantum Access MAX) with a heated-electrospray ionization (HESI-II) probe operated under negative ion and selected reaction monitoring (SRM) transitions (see Table S1 for parent and product ions).

50 µl of extract were injected and load into the TFC columns using 1% HCOOH eluent at 2000 mL/min. Analytes are then eluted by a MeOH plug and analyzed by the following gradient method at 0.3 mL/min using LC-MS grade methanol (mobile phase B) and 2 mM ammonium acetate/5% MEOH (mobile phase A): the initial solvent composition was 95 % A and 5 % B. Initial condition was hold for 1 min, , increased to 70 % B by 4 min, 100 % B by 9 min, hold until 12.5 min, then returned to initial conditions by 1 min and equilibrate for 10 min before next run.

In order to delay the interfering background peaks of PFAS, which can be present in solvents or released by the analytical system, a trap column (Thermo Hypersil GOLD 1.9 µm, 50 2.1 mm) was placed between the analytical pump and the injection valve.

*QA/QC*

Quantification was performed by isotopic dilution method and calibration curves were acquired in each analytical run. Calibration curve standards (0-100 µg/L) were daily prepared diluting certified analytical mixed standard solutions with acetonitrile, which were acidified to pH 3 and spiked with IS by adding 50 µL of concentrated formic acid and 100 µL of the IS solution (40 µg L−1) to 0.9 mL of mixed standard solution.

Procedural blanks were run for every extraction batch; their PFAS concentrations were always below respective LODs. Limits of detection (LODs) and limits of quantification (LOQs) in fish tissue were estimated according to International Organisation for 168 Standardisation Standard 6107- 2:2006 as, respectively, 3- fold and 10- fold the standard deviation of an extract of biological tissue fortified at 1 µg/L. The limits of detection (LODs) and limits of quantification (LOQs) values were 0.01 to 0.05 and 0.02 to 0.15 ng/g dw, respectively.

**Table 4.1.** Target analytes measured in the present study including monitoring parent ions, product ions, acronyms, corresponding internal standard (IS) and names of analytes. n/a not applicable.

| **Target analytes** | **Acronym** | **Parent ion (*m/z*)** | **Product ions (*m/z*)** | **Internal standard IS** |
|---|---|---|---|---|
| Perfluorohexanoate | PFHxA | 312.9 | 119.1, 268.9 | $^{13}$C2-PFHxA |
| Perfluoroheptanoate | PFHpA | 362.9 | 169.0, 318.9 | $^{13}$C4-PFOA |
| Perfluorooctanoate | PFOA | 412.9 | 169.0, 368.9 | $^{13}$C4-PFOA |
| Perfluorononanoate | PFNA | 462.9 | 218.9, 418.9 | $^{13}$C5-PFNA |
| Perfluorodecanoate | PFDA | 512.9 | 268.9, 468.9 | $^{13}$C2-PFDA |
| Perfluoroundecanoate | PFUnDA | 562.9 | 268.8, 518.8 | $^{13}$C2-PFUnDA |
| Perfluorododecanoate | PFDoDA | 612.9 | 318.8, 568.9 | $^{13}$C2-PFDoDA |
| Perfluorotridecanoate | PFTrDA | 662.9 | 369.0, 619.0 | $^{13}$C2-PFDoDA |
| Perfluorotetradecanoate | PFTeDA | 712.9 | 419.0, 669.0 | $^{13}$C2-PFDoDA |
| Perfluorobutane sulfonate | PFBS | 298.9 | 80.2, 99.1 | $^{13}$C2-PFHxA |
| Perfluorohexane sulfonate | PFHxS | 398.9 | 80.1, 99.0 | $^{18}$O2-PFHxS |
| Perfluorooctane sulfonate | PFOS | 498.9 | 80.3, 99.1 | $^{13}$C4-PFOS |
| Perfluorodecane sulfonate | PFDS | 598.9 | 80.0, 99,0 | $^{13}$C2-PFUnDA |
| Perfluoro-1-octanesulfonamide | FOSA | 497.9 | 78.0 | d-N-EtFOSA |
| 2,3,3,3-Tetrafluoro-2-(1,1,2,2,3,3,3-heptafluoroproproxy)propanoate | HPFO-DA, GenX | 329,0 | 169.0, 285,0, 185.0 | $^{13}$C4-PFOA |
| perfluoro([5-methoxy-1,3-dioxolan-4-yl]oxy)acetate | C6O4 | 339.0 | 85.0, 113.0 | $^{13}$C4-PFOA |
| dodecafluoro-3H-4,8-dioxanonanoate | ADONA | 377.0 | 85.0, 251.0 | $^{13}$C4-PFOA |
| 1H,1H,2H,2H-perfluorooctanesulphonate | 6:2 FTS | 427.0 | 407.0 | $^{13}$C4-PFOA |
| 1H,1H,2H,2H-perfluorodecanesulphonate | 8:2 FTS | 527.0 | 507.0 | $^{13}$C2-PFDA |
| Perfluoro-n-[$^{13}$C2] hexanoate | $^{13}C_2$-PFHxA | 314.9 | 269.9 | n/a |
| Perfluoro-n-[$^{13}$C4] octanoate | $^{13}C_4$-PFOA | 416.9 | 371.9 | n/a |
| Perfluoro-n-[$^{13}$C5] nonanoate | $^{13}C_5$-PFNA | 467.9 | 422.9 | n/a |
| Perfluoro-n-$^{[13}$C2] decanoate | $^{13}C_2$-PFDA | 514.9 | 469.9 | n/a |
| Perfluoro-n-[$^{13}$C2] undecanoate | $^{13}C_2$-PFUnDA | 564.9 | 519.8 | n/a |
| Perfluoro-n-[$^{13}$C2] dodecanoate | $^{13}C_2$-PFDoDA | 614.9 | 569.9 | n/a |
| Perfluoro-n-hexane [$^{18}$O2] sulfonate | $^{18}O_2$-PFHxS | 402.9 | 103.0 | n/a |
| Perfluoro-n-octane [$^{13}$C4] sulfonate | $^{13}C_4$-PFOS | 502.9 | 99.1 | n/a |
| N-ethyl-[d5]-perfluorooctane sulfonamide | d-N-EtFOSA | 531.0 | 169.0 | n/a |

#### 4.3.1.4. Statistical analysis

The heavy metals data obtained by the feathers analysis were processed by statistical analysis using the non-parametric test Kruskal-Wallis to highlight the differences between groups and between the two sampling years through the free software Past4.03. The same software was also used to investigate possible correlations between biomarkers and heavy metals Descriptive statistics have been elaborated through Microsoft excel.

Statistical analyses for POPs were performed using R-4.3.1 software (www.r-project.org). Prior to analysis, a logarithmic transformation was applied to the data, which exhibited a non-normal distribution based on the Shapiro-Wilk test ($p < 0.05$). To assess differences in concentrations among locations, Kruskal–Wallis one-way analysis of variance ($p< 0.05$) followed by Dunn's Test for multiple comparisons ($p<0.05$) were employed. PCA was applied to reveal the presence of distinctive patterns within the distributions of the target chemicals. Prior to PCA, all data were log-transformed. No further normalization or scaling transformations were applied.

The PFASs data obtained by the eggs analysis were processed by statistical analysis using the non-parametric test Kruskal-Wallis to investigate differences between groups, through the free software Past4.03. Due to the small sample size difference between sampling years were not evaluated. Descriptive statistics have been elaborated through Microsoft excel.

### 4.3.2. Results and discussion

#### 4.3.2.1. Heavy metals levels in feathers

The analysis of Cd, Pb, Hg and Cu concentrations was carried out on 42 samples, 18 from the year 2021 and 25 from 2022. As reported above, feathers were collected from nestlings sampled in the study areas (wood, urban, agri 1 and agri 2), during 2021 and 2022.

Regarding sampling year 2021 (Figure 4.3), Hg levels were significantly higher in urban nestlings compared to wood nestlings, whereas Pb and Cu showed the highest values in agri 2 individuals. Cd levels did not show differences between study areas.

In 2022 sampling year (Figure 4.3.), nestlings from urban, agri 1 and agri 2 areas showed levels of Hg significantly higher than individuals from wood. A statistically significant difference emerged also for Pb levels that resulted higher in urban and agri 2 areas compared to wood area. Urban and agri 2 nestlings showed also higher levels of Pb ($p<0.05$) than agri 1. Cd and Cu did not show any significant difference between areas, however levels or Cu were again higher in urban area.

Comparing the sampling years 2021 and 2022, each area no significant differences were found for none of the metals analysed.

In both years, all sites showed Cu feather concentrations one order of magnitude or higher than other analysed elements. For this reason, Cu results are reported on a different graph in Figure 4.4.

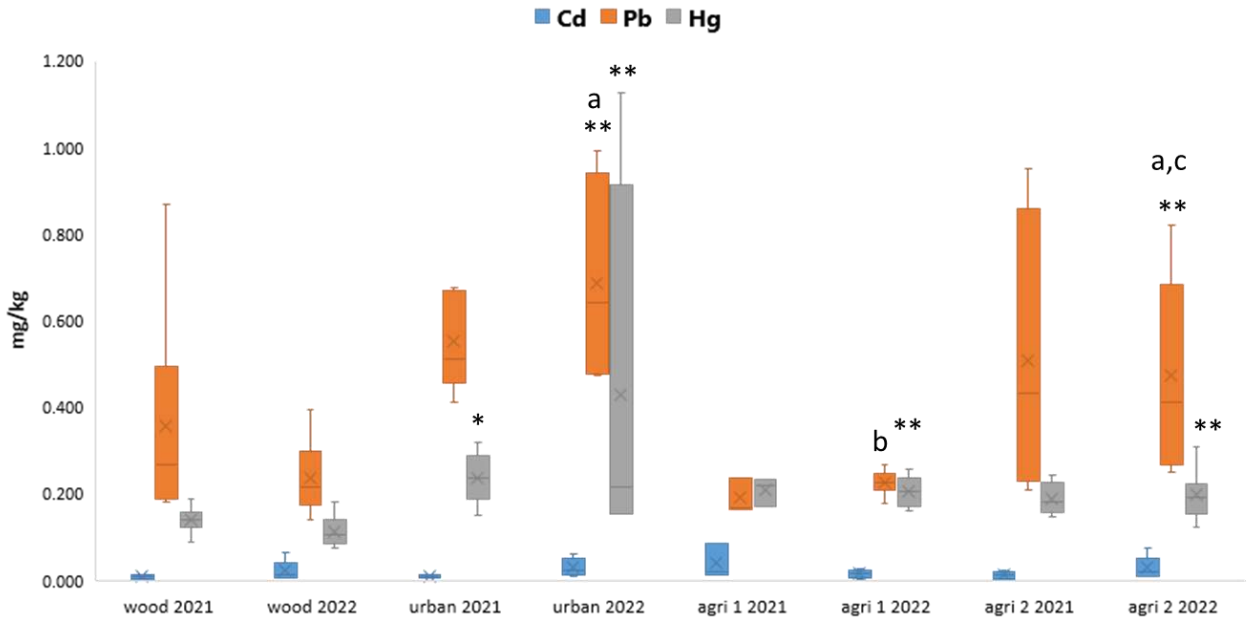


**Figure 4.3.** Levels of Cd, Pb and Hg measured in feathers samples of *Parus major* from the different study areas in 2021 and 2022. * indicates a statistically significant difference respect to wood for 2021, ** indicate a statistically significant difference respect to wood for 2022, different letters indicate a statistically significant difference among areas ($p<0.05$).

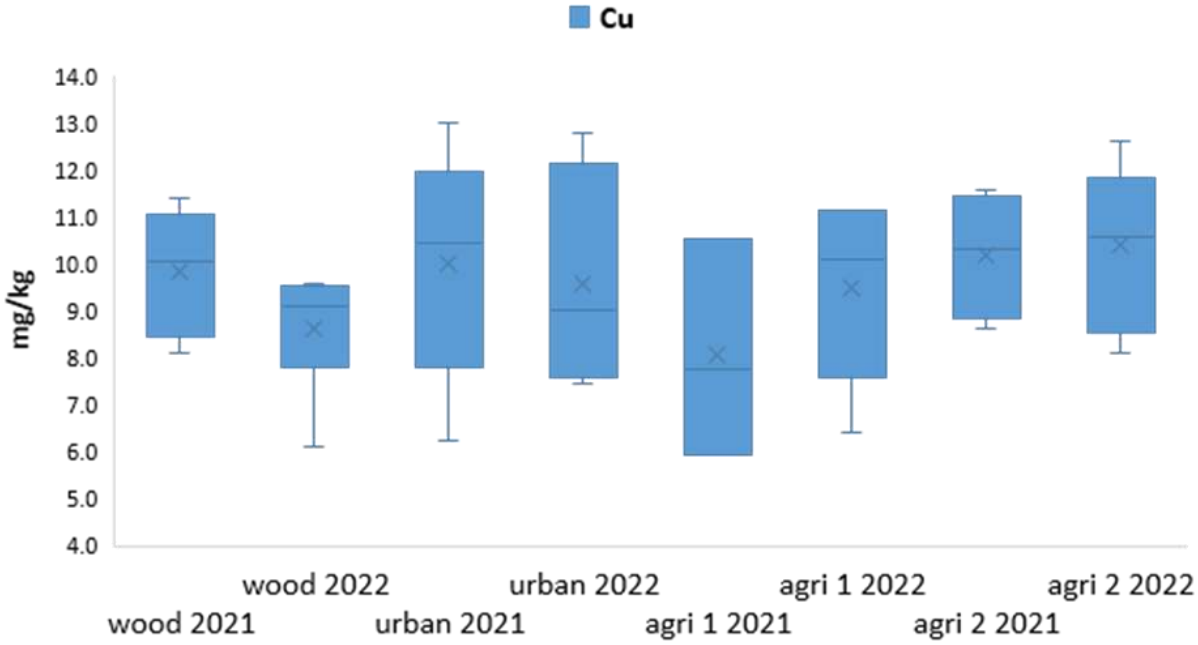


**Figure 4.4.** Levels of Cu measured in feathers samples of *Parus major* from the different study areas in 2021 and 2022.

The study of Costa and collaborators (2013) showed similar elevated values of Cu (8.29±1.75 ppm at the reference site and 9.11±3.67 ppm in the area close to the pulp mill) compared to Cd, Hg and Pb concentrations. High levels of Cu may be caused by significant anthropogenic inputs especially agricultural practices and atmospheric deposition. Even though copper is an important essential element, elevated concentration may cause toxic effects (Gaetke, 2003). Observing results of Costa et al., (2013) almost no difference in Cd, Hg and Pb concentrations were observed between the two study areas. The values are in line with the results obtained from feather samples of this study, where Cd levels range between 0.01 mg/kg and 0.07 mg/kg, Pb between 0.14 mg/kg and 0.99 mg/kg and Hg between 0.08 mg/kg and 1.13 mg/kg.

Markowski and collaborators (2014) examined heavy metal concentrations in feathers of nestling great tits, from an urban parkland and a deciduous forest. They found that Cd concentrations, from the parkland site, were very similar to the ones from the forest site. Their values are slightly higher than the ones detected in the areas in Veneto.

Significant differences in metal levels have been instead observed between two study sites in the work of Ding and collaborators (2023) in a free-living resident passerine bird, the tree sparrow *Passer montanus.* There were two study sites: a moderately polluted area that has been contaminated by industrial wastewater from metal smelting for a long time, with Cu, Zn, Pb, and Cd close to the city Baiyin and a relatively unpolluted site near Liujiaxia, chosen as the control site. The heavy metal levels in feathers of juvenile sparrows were significantly higher in the contaminated area, Cu was 22 mg/kg, Pb was about 18 mg/kg and Cd was 2 mg/kg. These values are much higher than the ones observed in Veneto, which can instead be comparable to those from the control site in Liujiaxia.

The results obtained in this study did not exceed the thresholds found by Burger and Gochfeld (2000), who analysed concentrations of metals in seabird feathers and stated that Cd concentrations over 2 ppm could have adverse effects. Whereas Pb levels of 4 ppm and Hg levels of 5 ppm in feathers are known to cause sublethal and reproductive effects.

All our results also are in line, or even lower, with the heavy metal concentrations of the control sites found in the above-mentioned studies. Therefore, it can be assumed that great tit specimens in the areas sampled in the Veneto region are not exposed to high heavy metal concentrations. Hg high concentrations found in the present study in urban samples were maybe related to local industrial and commercial emissions (Siudek et al., 2016). Furthermore, high levels of Pb in urban area could be due to higher traffic generally found close to cities (Levin et al., 2021).

#### 4.3.2.2. POPs levels in eggs

The presence of POPs contaminants was evaluated in a total of 20 unhatched and abandoned eggs of great tits sampled in the study areas during 2021 and 2022.

Concentration data were expressed in ng/g ww (wet weight) and ng/g lw (lipid weight). Detected ∑OCPs concentrations ranged from 10.1 to 292 ng/g ww, with a median of 114 ng/g ww (Table 4.2.), and from 340 to 8590 ng/g lw, with a median of 1730 ng/g lw (Table 4.3.). Significant higher levels were detected for all chemicals in lw compared to ww. Statistically significant differences in ΣOCPs concentrations (both ww and lw) were observed between locations (Kruskal–Wallis test and Dunn's Test, $p < 0.05$; Table 4.5.; Table 4.6). For ng/g ww concentrations, all OCPs families, except ∑HCHs, exhibited significant differences between locations, whereas for ng/g lw concentrations, only ∑DDTs showed a significant difference between locations. The order of highest median ∑OCPs concentrations for all families was Urban > Agri 1 > Wood > Agri 2. Notably, in tit eggs from all locations, the relative abundance based on median concentrations followed this order: ∑DDTs > HxCB > ∑HCHs > PeCB > ∑endosulfan (Figure 4.5; Figure 4.8.).

The primary contribution to the ∑OCPs (median: 114 ng/g ww) profiles is mainly attributed to ∑DDTs (median: 113 ng/g ww), primarily due to elevated levels of pp'-DDE (median: 112 ng/g ww) (Table 4.2.; Table 4.3.). Similar to ∑OCPs, the highest median levels of ∑DDTs followed the same order. All DDTs isomers exhibited significant differences between locations for ng/g ww concentrations, except for op'-DDE, whereas for ng/g lw concentrations, only pp'-DDE showed significant differences between locations (Kruskal–Wallis test and Dunn's Test, $p < 0.05$; Table 4.5.; Table 4.6.). HxCB depicted a relatively high contribution, particularly in the Wood location (Figure 4.6.; Figure 4.8.). Despite the Urban location being the most polluted (median: 1.28 ng/g ww), the Wood location closely followed (median: 1.02 ng/g ww) (Table 4.2.; Table 4.3.) without significant differences between them (Table 4.7; Table 4.8.). Statistically significant differences were observed for PeCB concentrations (ng/g ww) between locations, especially in the Urban location, which differed significantly from all other locations due to its high concentrations (Kruskal–Wallis test and Dunn's Test, $p < 0.05$; Table 4.5.; Table 4.7.).The highest median levels of ∑HCHs were detected in the Urban location. The HCHs isomer profile varied between locations, with the Urban location being dominated by the γ-HCH similar to Agri 2, while the Wood and Agri 1 locations were dominated by β-HCH (Figure 4.6.; Figure 4.8.). The contribution of ∑endosulfan to the OCPs profile was minimal, primarily attributed to α-endosulfan.

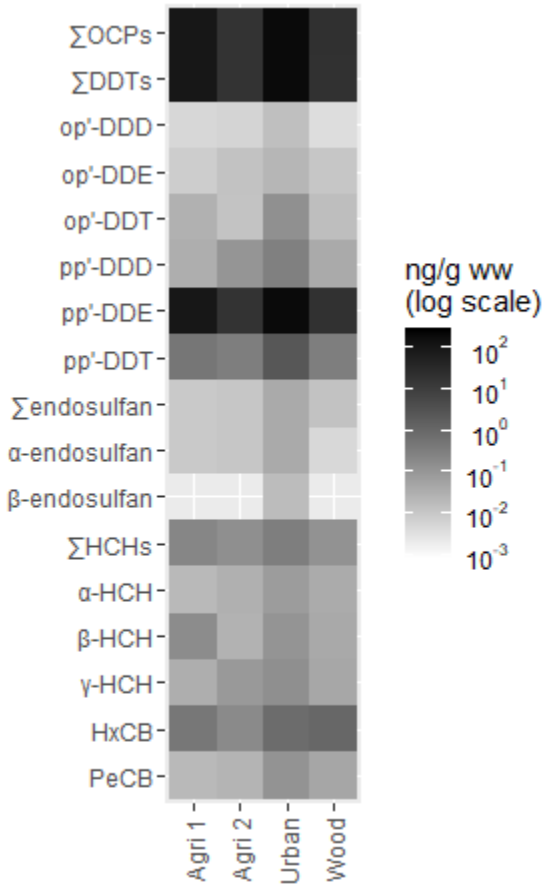


**Figure 4.5.** Chemical distribution (ng/g ww) in logarithmic scale across study areas.

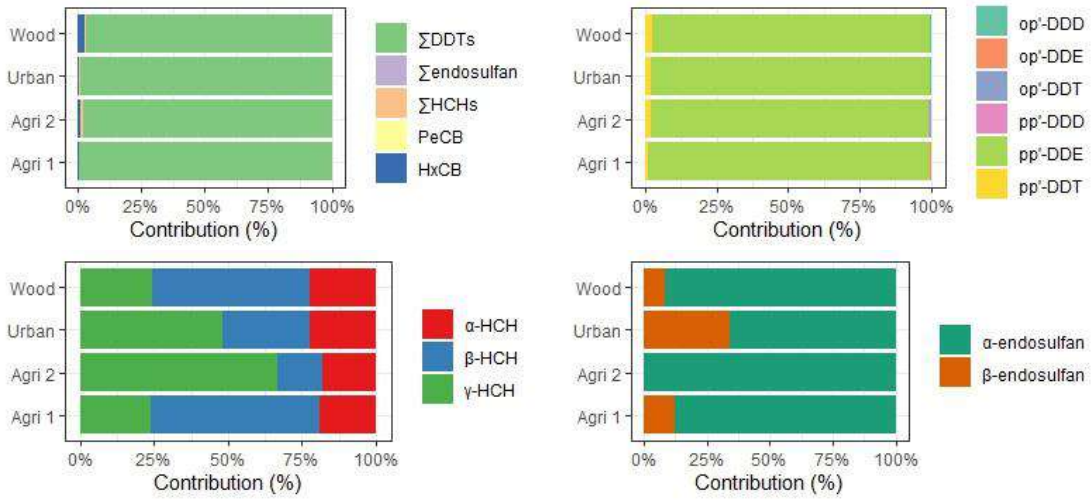


**Figure 4.6.** Contribution of pesticide families (ng/g ww) in study areas (top-left), of DDT isomers (top-right), of HCH isomers (bottom-left) and endosulfan isomers (bottom-right).

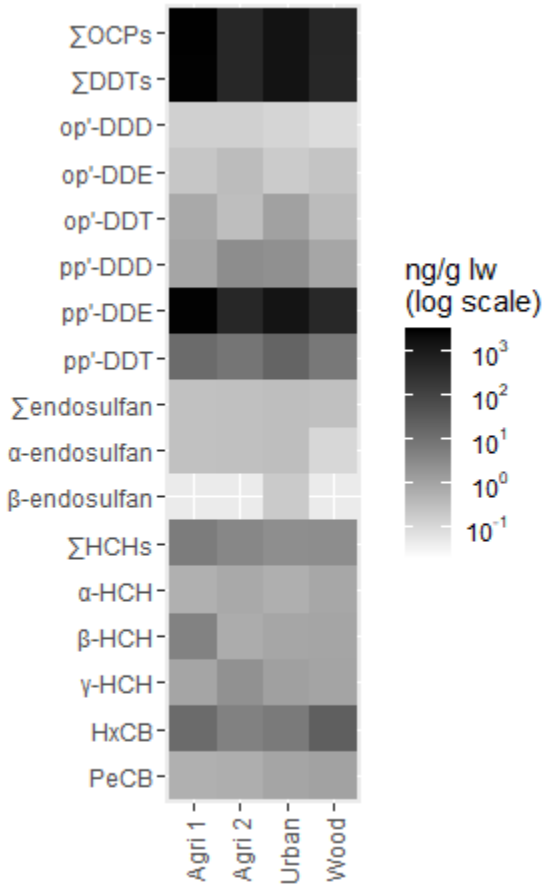


**Figure 4.7.** Chemical distribution (ng/g lw) in logarithmic scale across study areas.

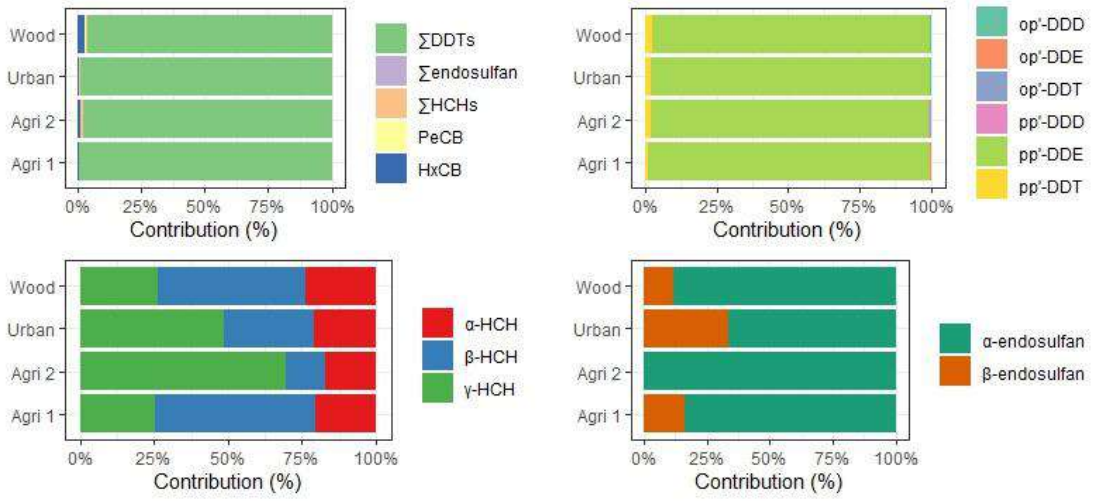


**Figure 4.8.** Contribution of pesticide families (ng/g lw) in study areas (top-left), of DDT isomers (top-right), of HCH isomers (bottom-left) and endosulfan isomers (bottom-right).

**Table 4.2.** Concentration in ng/g ww of the contaminants.

| Study area | ng/g ww | PeCB | HxCB | α-HCH | γ-HCH | β-HCH | ∑HCHs | α-endosulfan | β-endosulfan | ∑endosulfan | op'-DDE | pp'-DDE | op'-DDD | op'-DDT | pp'-DDD | pp'-DDT | ∑DDTs | ∑OCPs |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Overall (n=20) | Mean | 0.0787 | 0.9717 | 0.0493 | 0.0877 | 0.0923 | 0.2293 | 0.0180 | 0.0059 | 0.0239 | 0.0137 | 122.0992 | 0.0075 | 0.0685 | 0.2713 | 1.9870 | 124.4472 | 125.7508 |
| | Median | 0.0606 | 0.8893 | 0.0367 | 0.0837 | 0.0633 | 0.2016 | 0.0133 | 0.0000 | 0.0140 | 0.0096 | 111.4875 | 0.0048 | 0.0393 | 0.1322 | 1.1121 | 112.7298 | 113.7989 |
| | Min | 0.0185 | 0.1054 | 0.0000 | 0.0000 | 0.0110 | 0.0612 | 0.0000 | 0.0000 | 0.0000 | 0.0000 | 9.6512 | 0.0000 | 0.0067 | 0.0236 | 0.1830 | 9.9054 | 10.1417 |
| | Max | 0.2014 | 2.1295 | 0.1721 | 0.2471 | 0.3318 | 0.5871 | 0.0407 | 0.0302 | 0.0669 | 0.0724 | 283.9118 | 0.0543 | 0.2312 | 1.1162 | 5.8232 | 289.7480 | 292.0955 |
| Wood (n=6) | Mean | 0.0662 | 1.1153 | 0.0516 | 0.0560 | 0.1239 | 0.2315 | 0.0152 | 0.0014 | 0.0165 | 0.0147 | 37.9061 | 0.0070 | 0.0366 | 0.1059 | 0.9961 | 39.0663 | 40.4959 |
| | Median | 0.0606 | 1.0205 | 0.0384 | 0.0302 | 0.0746 | 0.1795 | 0.0091 | 0.0000 | 0.0119 | 0.0138 | 30.1210 | 0.0073 | 0.0393 | 0.0760 | 0.5311 | 30.7729 | 32.1585 |
| | Min | 0.0498 | 0.7490 | 0.0261 | 0.0000 | 0.0428 | 0.0961 | 0.0000 | 0.0000 | 0.0000 | 0.0040 | 22.0426 | 0.0027 | 0.0152 | 0.0236 | 0.3450 | 22.4590 | 23.7939 |
| | Max | 0.0918 | 1.5978 | 0.1041 | 0.1851 | 0.3318 | 0.4546 | 0.0351 | 0.0082 | 0.0351 | 0.0258 | 71.3302 | 0.0107 | 0.0677 | 0.2818 | 2.4970 | 74.2133 | 75.6740 |
| Urban (n=7) | Mean | 0.1389 | 1.3345 | 0.0703 | 0.1530 | 0.0929 | 0.3162 | 0.0282 | 0.0145 | 0.0427 | 0.0178 | 213.3165 | 0.0115 | 0.1454 | 0.5105 | 3.9114 | 217.9130 | 219.7453 |
| | Median | 0.1290 | 1.2769 | 0.0605 | 0.1441 | 0.0713 | 0.3119 | 0.0318 | 0.0174 | 0.0407 | 0.0046 | 203.5436 | 0.0000 | 0.1462 | 0.3325 | 3.4697 | 206.6863 | 208.0747 |
| | Min | 0.0980 | 0.8644 | 0.0000 | 0.0921 | 0.0271 | 0.1192 | 0.0093 | 0.0000 | 0.0132 | 0.0000 | 112.2554 | 0.0000 | 0.0564 | 0.1817 | 1.6372 | 114.1439 | 115.5237 |
| | Max | 0.2014 | 2.1295 | 0.1721 | 0.2471 | 0.1679 | 0.5871 | 0.0407 | 0.0302 | 0.0669 | 0.0724 | 283.9118 | 0.0543 | 0.2312 | 1.1162 | 5.8232 | 289.7480 | 292.0955 |
| Agri 1 (n=5) | Mean | 0.0320 | 0.6200 | 0.0275 | 0.0349 | 0.0829 | 0.1452 | 0.0114 | 0.0016 | 0.0130 | 0.0083 | 138.5194 | 0.0039 | 0.0228 | 0.2094 | 1.1701 | 139.9339 | 140.7441 |
| | Median | 0.0303 | 0.4997 | 0.0262 | 0.0374 | 0.0532 | 0.1302 | 0.0129 | 0.0000 | 0.0129 | 0.0081 | 124.0746 | 0.0044 | 0.0263 | 0.0606 | 0.4337 | 124.4873 | 125.6871 |
| | Min | 0.0206 | 0.3551 | 0.0080 | 0.0000 | 0.0282 | 0.0612 | 0.0058 | 0.0000 | 0.0058 | 0.0012 | 109.6805 | 0.0008 | 0.0081 | 0.0303 | 0.3125 | 110.0686 | 111.0780 |
| | Max | 0.0540 | 1.0177 | 0.0466 | 0.0519 | 0.1734 | 0.2283 | 0.0148 | 0.0082 | 0.0218 | 0.0153 | 212.7599 | 0.0073 | 0.0362 | 0.7954 | 4.2362 | 217.8502 | 218.3877 |
| Agri 2 (n=2) | Mean | 0.0218 | 0.1503 | 0.0236 | 0.0859 | 0.0193 | 0.1287 | 0.0077 | 0.0000 | 0.0077 | 0.0098 | 14.3679 | 0.0035 | 0.0096 | 0.0850 | 0.2669 | 14.7427 | 15.0512 |
| | Median | 0.0218 | 0.1503 | 0.0236 | 0.0859 | 0.0193 | 0.1287 | 0.0077 | 0.0000 | 0.0077 | 0.0098 | 14.3679 | 0.0035 | 0.0096 | 0.0850 | 0.2669 | 14.7427 | 15.0512 |
| | Min | 0.0185 | 0.1054 | 0.0166 | 0.0806 | 0.0110 | 0.1081 | 0.0042 | 0.0000 | 0.0042 | 0.0062 | 9.6512 | 0.0012 | 0.0067 | 0.0571 | 0.1830 | 9.9054 | 10.1417 |
| | Max | 0.0250 | 0.1951 | 0.0305 | 0.0912 | 0.0276 | 0.1493 | 0.0112 | 0.0000 | 0.0112 | 0.0135 | 19.0846 | 0.0057 | 0.0126 | 0.1129 | 0.3508 | 19.5800 | 19.9606 |

**Table 4.3**. Concentration in ng/g lw of the contaminants.

| Study area | ng/g lw | PeCB | HxCB | α-HCH | γ-HCH | β-HCH | ∑HCHs | α-endosulfan | β-endosulfan | ∑endosulfan | op'-DDE | pp'-DDE | op'-DDD | op'-DDT | pp'-DDD | pp'-DDT | ∑DDTs | ∑OCPs |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Overall (n=20) | Mean | 1.084 | 14.634 | 0.761 | 1.372 | 1.476 | 3.609 | 0.257 | 0.067 | 0.324 | 0.212 | 1888.649 | 0.102 | 0.817 | 4.076 | 27.103 | 1920.959 | 1940.610 |
| | Median | 1.076 | 14.072 | 0.721 | 1.069 | 1.086 | 3.349 | 0.244 | 0.000 | 0.305 | 0.209 | 1674.804 | 0.087 | 0.616 | 2.961 | 17.655 | 1713.939 | 1729.794 |
| | Min | 0.514 | 5.034 | 0.000 | 0.000 | 0.263 | 1.159 | 0.000 | 0.000 | 0.000 | 0.000 | 309.060 | 0.000 | 0.190 | 0.330 | 5.837 | 316.009 | 340.703 |
| | Max | 2.208 | 25.991 | 1.882 | 4.612 | 4.917 | 6.780 | 0.536 | 0.321 | 0.858 | 0.601 | 8371.694 | 0.353 | 2.499 | 31.298 | 166.686 | 8571.988 | 8593.137 |
| Wood (n=6) | Mean | 1.112 | 19.201 | 0.879 | 0.981 | 1.863 | 3.723 | 0.222 | 0.030 | 0.252 | 0.234 | 609.287 | 0.110 | 0.569 | 1.643 | 15.074 | 626.917 | 651.206 |
| | Median | 1.135 | 20.386 | 0.762 | 0.690 | 1.462 | 2.968 | 0.167 | 0.000 | 0.239 | 0.233 | 628.191 | 0.110 | 0.557 | 1.598 | 11.516 | 642.186 | 670.208 |
| | Min | 0.736 | 11.241 | 0.458 | 0.000 | 0.887 | 1.461 | 0.000 | 0.000 | 0.000 | 0.098 | 309.060 | 0.066 | 0.356 | 0.330 | 5.837 | 316.009 | 340.703 |
| | Max | 1.405 | 25.723 | 1.882 | 3.346 | 4.231 | 6.780 | 0.467 | 0.182 | 0.467 | 0.367 | 909.741 | 0.152 | 0.863 | 3.595 | 31.847 | 946.512 | 965.140 |
| Urban (n=7) | Mean | 1.261 | 11.869 | 0.581 | 1.343 | 0.831 | 2.755 | 0.235 | 0.120 | 0.355 | 0.122 | 1789.551 | 0.077 | 1.252 | 3.984 | 31.993 | 1826.980 | 1843.220 |
| | Median | 0.952 | 12.415 | 0.654 | 1.187 | 0.900 | 2.701 | 0.201 | 0.188 | 0.342 | 0.050 | 1795.610 | 0.000 | 1.192 | 3.233 | 31.984 | 1832.521 | 1847.368 |
| | Min | 0.844 | 5.615 | 0.000 | 0.828 | 0.263 | 1.159 | 0.138 | 0.000 | 0.168 | 0.000 | 1586.429 | 0.000 | 0.458 | 2.508 | 20.549 | 1610.923 | 1621.744 |
| | Max | 2.208 | 17.559 | 1.118 | 2.224 | 1.678 | 4.557 | 0.414 | 0.204 | 0.601 | 0.470 | 2046.094 | 0.353 | 2.499 | 7.251 | 39.311 | 2089.156 | 2111.046 |
| Agri 1 (n=5) | Mean | 0.892 | 16.666 | 0.831 | 1.037 | 2.235 | 4.104 | 0.326 | 0.064 | 0.390 | 0.255 | 4109.087 | 0.123 | 0.692 | 7.520 | 41.627 | 4159.303 | 4181.356 |
| | Median | 0.790 | 14.171 | 0.614 | 0.974 | 1.293 | 4.492 | 0.330 | 0.000 | 0.330 | 0.222 | 3168.677 | 0.113 | 0.671 | 1.548 | 10.544 | 3179.218 | 3209.858 |
| | Min | 0.514 | 11.135 | 0.195 | 0.000 | 0.720 | 1.488 | 0.141 | 0.000 | 0.141 | 0.029 | 2574.291 | 0.020 | 0.190 | 0.711 | 7.982 | 2583.400 | 2607.091 |
| | Max | 1.379 | 25.991 | 1.836 | 2.042 | 4.917 | 6.474 | 0.536 | 0.321 | 0.858 | 0.601 | 8371.694 | 0.286 | 1.423 | 31.298 | 166.686 | 8571.988 | 8593.137 |
| Agri 2 (n=2) | Mean | 0.852 | 5.535 | 0.868 | 3.484 | 0.670 | 5.022 | 0.265 | 0.000 | 0.265 | 0.351 | 522.487 | 0.109 | 0.353 | 3.092 | 9.763 | 536.155 | 547.830 |
| | Median | 0.852 | 5.535 | 0.868 | 3.484 | 0.670 | 5.022 | 0.265 | 0.000 | 0.265 | 0.351 | 522.487 | 0.109 | 0.353 | 3.092 | 9.763 | 536.155 | 547.830 |
| | Min | 0.645 | 5.034 | 0.788 | 2.355 | 0.628 | 3.854 | 0.241 | 0.000 | 0.241 | 0.349 | 492.529 | 0.071 | 0.324 | 2.913 | 9.053 | 505.314 | 515.137 |
| | Max | 1.060 | 6.036 | 0.949 | 4.612 | 0.711 | 6.189 | 0.290 | 0.000 | 0.290 | 0.354 | 552.444 | 0.147 | 0.382 | 3.271 | 10.473 | 566.996 | 580.522 |

**Table 4.4.** Wilcoxon rank sum test (p<0.05). In bold those that shows significant differences between ng/g lw vs ng/g ww.

| Chemical | statistic | p.value | method | alternative | effectsize | estimate | conf.level | conf.low | conf.high | conf.method | n.obs |
|---|---|---|---|---|---|---|---|---|---|---|---|
| **OCP** | **400** | **0.00000** | **Wilcoxon rank sum test** | **two.sided** | **r (rank biserial)** | **1.00** | **0.95** | **1.00** | **1.00** | **normal** | **40** |
| **DDT** | **400** | **0.00000** | **Wilcoxon rank sum test** | **two.sided** | **r (rank biserial)** | **1.00** | **0.95** | **1.00** | **1.00** | **normal** | **40** |
| **opDDE** | **287** | **0.00000** | **Wilcoxon rank sum test** | **two.sided** | **r (rank biserial)** | **0.99** | **0.95** | **0.97** | **0.99** | **normal** | **34** |
| **ppDDE** | **400** | **0.00000** | **Wilcoxon rank sum test** | **two.sided** | **r (rank biserial)** | **1.00** | **0.95** | **1.00** | **1.00** | **normal** | **40** |
| **opDDD** | **254** | **0.00000** | **Wilcoxon rank sum test** | **two.sided** | **r (rank biserial)** | **0.98** | **0.95** | **0.97** | **0.99** | **normal** | **32** |
| **ppDDD** | **391** | **0.00000** | **Wilcoxon rank sum test** | **two.sided** | **r (rank biserial)** | **0.96** | **0.95** | **0.91** | **0.98** | **normal** | **40** |
| **opDDT** | **397** | **0.00000** | **Wilcoxon rank sum test** | **two.sided** | **r (rank biserial)** | **0.99** | **0.95** | **0.97** | **0.99** | **normal** | **40** |
| **ppDDT** | **400** | **0.00000** | **Wilcoxon rank sum test** | **two.sided** | **r (rank biserial)** | **1.00** | **0.95** | **1.00** | **1.00** | **normal** | **40** |
| **Endosulfan** | **361** | **0.00000** | **Wilcoxon rank sum test** | **two.sided** | **r (rank biserial)** | **1.00** | **0.95** | **1.00** | **1.00** | **normal** | **38** |
| **EndosulfanI** | **361** | **0.00000** | **Wilcoxon rank sum test** | **two.sided** | **r (rank biserial)** | **1.00** | **0.95** | **1.00** | **1.00** | **normal** | **38** |
| **EndosulfanII** | **49** | **0.00217** | **Wilcoxon rank sum test** | **two.sided** | **r (rank biserial)** | **1.00** | **0.95** | **1.00** | **1.00** | **normal** | **14** |
| **HCH** | **400** | **0.00000** | **Wilcoxon rank sum test** | **two.sided** | **r (rank biserial)** | **1.00** | **0.95** | **1.00** | **1.00** | **normal** | **40** |
| **alfa** | **324** | **0.00000** | **Wilcoxon rank sum test** | **two.sided** | **r (rank biserial)** | **1.00** | **0.95** | **1.00** | **1.00** | **normal** | **36** |
| **beta** | **398** | **0.00000** | **Wilcoxon rank sum test** | **two.sided** | **r (rank biserial)** | **0.99** | **0.95** | **0.98** | **1.00** | **normal** | **40** |
| **gamma** | **313** | **0.00000** | **Wilcoxon rank sum test** | **two.sided** | **r (rank biserial)** | **0.93** | **0.95** | **0.86** | **0.97** | **normal** | **36** |
| **HCB** | **400** | **0.00000** | **Wilcoxon rank sum test** | **two.sided** | **r (rank biserial)** | **1.00** | **0.95** | **1.00** | **1.00** | **normal** | **40** |
| **PeCB** | **400** | **0.00000** | **Wilcoxon rank sum test** | **two.sided** | **r (rank biserial)** | **1.00** | **0.95** | **1.00** | **1.00** | **normal** | **40** |

**Table 4.5.** Kruskal–Wallis one-way analysis of variance (p<0.05). In bold those that shows significant differences in concentrations (ng/g ww) among locations.

| Chemical | statistic | df.error | p.value | method | effectsize | estimate | conf.level | conf.low | conf.high | conf.method | conf.iterations | n.obs |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **OCP** | **15.70** | **3** | **0.0013** | **Kruskal-Wallis rank sum test** | **Epsilon2 (rank)** | **0.83** | **0.95** | **0.77** | **1** | **percentile bootstrap** | **100** | **20** |
| **DDT** | **15.70** | **3** | **0.0013** | **Kruskal-Wallis rank sum test** | **Epsilon2 (rank)** | **0.83** | **0.95** | **0.77** | **1** | **percentile bootstrap** | **100** | **20** |
| opDDE | 3.97 | 3 | 0.2647 | Kruskal-Wallis rank sum test | Epsilon2 (rank) | 0.25 | 0.95 | 0.07 | 1 | percentile bootstrap | 100 | 17 |
| **ppDDE** | **15.70** | **3** | **0.0013** | **Kruskal-Wallis rank sum test** | **Epsilon2 (rank)** | **0.83** | **0.95** | **0.77** | **1** | **percentile bootstrap** | **100** | **20** |
| **opDDD** | **8.86** | **3** | **0.0312** | **Kruskal-Wallis rank sum test** | **Epsilon2 (rank)** | **0.59** | **0.95** | **0.49** | **1** | **percentile bootstrap** | **100** | **16** |
| **ppDDD** | **8.88** | **3** | **0.0310** | **Kruskal-Wallis rank sum test** | **Epsilon2 (rank)** | **0.47** | **0.95** | **0.34** | **1** | **percentile bootstrap** | **100** | **20** |
| **opDDT** | **15.22** | **3** | **0.0016** | **Kruskal-Wallis rank sum test** | **Epsilon2 (rank)** | **0.80** | **0.95** | **0.72** | **1** | **percentile bootstrap** | **100** | **20** |
| **ppDDT** | **11.20** | **3** | **0.0107** | **Kruskal-Wallis rank sum test** | **Epsilon2 (rank)** | **0.59** | **0.95** | **0.44** | **1** | **percentile bootstrap** | **100** | **20** |
| **Endosulfan** | **10.44** | **3** | **0.0152** | **Kruskal-Wallis rank sum test** | **Epsilon2 (rank)** | **0.58** | **0.95** | **0.36** | **1** | **percentile bootstrap** | **100** | **19** |
| EndosulfanI | 6.06 | 3 | 0.1088 | Kruskal-Wallis rank sum test | Epsilon2 (rank) | 0.34 | 0.95 | 0.15 | 1 | percentile bootstrap | 100 | 19 |
| EndosulfanII | 1.46 | 2 | 0.4826 | Kruskal-Wallis rank sum test | Epsilon2 (rank) | 0.24 | 0.95 | 0.23 | 1 | percentile bootstrap | 100 | 7 |
| HCH | 4.74 | 3 | 0.1917 | Kruskal-Wallis rank sum test | Epsilon2 (rank) | 0.25 | 0.95 | 0.13 | 1 | percentile bootstrap | 100 | 20 |
| **alfa** | **10.15** | **3** | **0.0173** | **Kruskal-Wallis rank sum test** | **Epsilon2 (rank)** | **0.60** | **0.95** | **0.50** | **1** | **percentile bootstrap** | **100** | **18** |
| beta | 5.07 | 3 | 0.1668 | Kruskal-Wallis rank sum test | Epsilon2 (rank) | 0.27 | 0.95 | 0.23 | 1 | percentile bootstrap | 100 | 20 |
| **gamma** | **9.96** | **3** | **0.0189** | **Kruskal-Wallis rank sum test** | **Epsilon2 (rank)** | **0.59** | **0.95** | **0.43** | **1** | **percentile bootstrap** | **100** | **18** |
| **HCB** | **10.94** | **3** | **0.0120** | **Kruskal-Wallis rank sum test** | **Epsilon2 (rank)** | **0.58** | **0.95** | **0.44** | **1** | **percentile bootstrap** | **100** | **20** |
| **PeCB** | **16.45** | **3** | **0.0009** | **Kruskal-Wallis rank sum test** | **Epsilon2 (rank)** | **0.87** | **0.95** | **0.82** | **1** | **percentile bootstrap** | **100** | **20** |

**Table 4.6.** Kruskal–Wallis one-way analysis of variance (p<0.05). In bold those that shows significant differences in concentrations (ng/g lw) among locations.

| Chemical | statistic | df.error | p.value | method | effectsize | estimate | conf.level | conf.low | conf.high | conf.method | conf.iterations | n.obs |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **OCP** | **16.89** | **3** | **0.0007** | **Kruskal-Wallis rank sum test** | **Epsilon2 (rank)** | **0.89** | **0.95** | **0.89** | **1** | **percentile bootstrap** | **100** | **20** |
| **DDT** | **16.79** | **3** | **0.0008** | **Kruskal-Wallis rank sum test** | **Epsilon2 (rank)** | **0.88** | **0.95** | **0.88** | **1** | **percentile bootstrap** | **100** | **20** |
| opDDE | 2.10 | 3 | 0.5519 | Kruskal-Wallis rank sum test | Epsilon2 (rank) | 0.13 | 0.95 | 0.08 | 1 | percentile bootstrap | 100 | 17 |
| **ppDDE** | **16.79** | **3** | **0.0008** | **Kruskal-Wallis rank sum test** | **Epsilon2 (rank)** | **0.88** | **0.95** | **0.88** | **1** | **percentile bootstrap** | **100** | **20** |
| opDDD | 0.38 | 3 | 0.9442 | Kruskal-Wallis rank sum test | Epsilon2 (rank) | 0.03 | 0.95 | 0.04 | 1 | percentile bootstrap | 100 | 16 |
| ppDDD | 6.25 | 3 | 0.1002 | Kruskal-Wallis rank sum test | Epsilon2 (rank) | 0.33 | 0.95 | 0.16 | 1 | percentile bootstrap | 100 | 20 |
| opDDT | 6.67 | 3 | 0.0831 | Kruskal-Wallis rank sum test | Epsilon2 (rank) | 0.35 | 0.95 | 0.19 | 1 | percentile bootstrap | 100 | 20 |
| ppDDT | 7.81 | 3 | 0.0500 | Kruskal-Wallis rank sum test | Epsilon2 (rank) | 0.41 | 0.95 | 0.32 | 1 | percentile bootstrap | 100 | 20 |
| Endosulfan | 0.92 | 3 | 0.8205 | Kruskal-Wallis rank sum test | Epsilon2 (rank) | 0.05 | 0.95 | 0.04 | 1 | percentile bootstrap | 100 | 19 |
| EndosulfanI | 1.43 | 3 | 0.6978 | Kruskal-Wallis rank sum test | Epsilon2 (rank) | 0.08 | 0.95 | 0.04 | 1 | percentile bootstrap | 100 | 19 |
| EndosulfanII | 2.83 | 2 | 0.2431 | Kruskal-Wallis rank sum test | Epsilon2 (rank) | 0.47 | 0.95 | 0.39 | 1 | percentile bootstrap | 100 | 7 |
| HCH | 3.66 | 3 | 0.3001 | Kruskal-Wallis rank sum test | Epsilon2 (rank) | 0.19 | 0.95 | 0.10 | 1 | percentile bootstrap | 100 | 20 |
| alfa | 0.72 | 3 | 0.8689 | Kruskal-Wallis rank sum test | Epsilon2 (rank) | 0.04 | 0.95 | 0.02 | 1 | percentile bootstrap | 100 | 18 |
| beta | 7.22 | 3 | 0.0651 | Kruskal-Wallis rank sum test | Epsilon2 (rank) | 0.38 | 0.95 | 0.23 | 1 | percentile bootstrap | 100 | 20 |
| gamma | 4.79 | 3 | 0.1880 | Kruskal-Wallis rank sum test | Epsilon2 (rank) | 0.28 | 0.95 | 0.23 | 1 | percentile bootstrap | 100 | 18 |
| HCB | 7.59 | 3 | 0.0553 | Kruskal-Wallis rank sum test | Epsilon2 (rank) | 0.40 | 0.95 | 0.29 | 1 | percentile bootstrap | 100 | 20 |
| PeCB | 2.73 | 3 | 0.4347 | Kruskal-Wallis rank sum test | Epsilon2 (rank) | 0.14 | 0.95 | 0.07 | 1 | percentile bootstrap | 100 | 20 |

**Table 4.7.** Dunn's Test for multiple comparisons (p<0.05). In bold those that shows significant differences in concentrations (ng/g ww) among locations.

| Chemical | group1 | group2 | statistic | p.value | alternative | distribution | p.adjust.method | test |
|---|---|---|---|---|---|---|---|---|
| **POP** | **Agri 1** | **Agri 2** | **2.16** | **0.0306** | **two.sided** | **z** | **None** | **Dunn** |
| POP | Agri 1 | Urban | 1.14 | 0.2550 | two.sided | z | None | Dunn |
| POP | Agri 1 | Wood | 1.87 | 0.0614 | two.sided | z | None | Dunn |
| **POP** | **Agri 2** | **Urban** | **3.09** | **0.0020** | **two.sided** | **z** | **None** | **Dunn** |
| POP | Agri 2 | Wood | 0.83 | 0.4076 | two.sided | z | None | Dunn |
| **POP** | **Urban** | **Wood** | **3.23** | **0.0012** | **two.sided** | **z** | **None** | **Dunn** |
| **DDT** | **Agri 1** | **Agri 2** | **2.16** | **0.0306** | **two.sided** | **z** | **None** | **Dunn** |
| DDT | Agri 1 | Urban | 1.14 | 0.2550 | two.sided | z | None | Dunn |
| DDT | Agri 1 | Wood | 1.87 | 0.0614 | two.sided | z | None | Dunn |
| **DDT** | **Agri 2** | **Urban** | **3.09** | **0.0020** | **two.sided** | **z** | **None** | **Dunn** |
| DDT | Agri 2 | Wood | 0.83 | 0.4076 | two.sided | z | None | Dunn |
| **DDT** | **Urban** | **Wood** | **3.23** | **0.0012** | **two.sided** | **z** | **None** | **Dunn** |
| opDDE | Agri 1 | Agri 2 | 0.24 | 0.8129 | two.sided | z | None | Dunn |
| opDDE | Agri 1 | Urban | 1.85 | 0.0650 | two.sided | z | None | Dunn |
| opDDE | Agri 1 | Wood | 1.31 | 0.1908 | two.sided | z | None | Dunn |
| opDDE | Agri 2 | Urban | 1.20 | 0.2299 | two.sided | z | None | Dunn |
| opDDE | Agri 2 | Wood | 0.73 | 0.4669 | two.sided | z | None | Dunn |
| opDDE | Urban | Wood | 0.69 | 0.4900 | two.sided | z | None | Dunn |
| **ppDDE** | **Agri 1** | **Agri 2** | **2.16** | **0.0306** | **two.sided** | **z** | **None** | **Dunn** |
| ppDDE | Agri 1 | Urban | 1.14 | 0.2550 | two.sided | z | None | Dunn |
| ppDDE | Agri 1 | Wood | 1.87 | 0.0614 | two.sided | z | None | Dunn |
| **ppDDE** | **Agri 2** | **Urban** | **3.09** | **0.0020** | **two.sided** | **z** | **None** | **Dunn** |
| ppDDE | Agri 2 | Wood | 0.83 | 0.4076 | two.sided | z | None | Dunn |
| **ppDDE** | **Urban** | **Wood** | **3.23** | **0.0012** | **two.sided** | **z** | **None** | **Dunn** |
| opDDD | Agri 1 | Agri 2 | 0.10 | 0.9200 | two.sided | z | None | Dunn |
| **opDDD** | **Agri 1** | **Urban** | **2.76** | **0.0058** | **two.sided** | **z** | **None** | **Dunn** |
| opDDD | Agri 1 | Wood | 1.25 | 0.2118 | two.sided | z | None | Dunn |
| **opDDD** | **Agri 2** | **Urban** | **2.30** | **0.0214** | **two.sided** | **z** | **None** | **Dunn** |
| opDDD | Agri 2 | Wood | 1.03 | 0.3035 | two.sided | z | None | Dunn |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| opDDD | Urban | Wood | 1.78 | 0.0747 | two.sided | z | None | Dunn |
| ppDDD | Agri 1 | Agri 2 | 0.20 | 0.8399 | two.sided | z | None | Dunn |
| **ppDDD** | **Agri 1** | **Urban** | **2.27** | **0.0233** | **two.sided** | **z** | **None** | **Dunn** |
| ppDDD | Agri 1 | Wood | 0.14 | 0.8890 | two.sided | z | None | Dunn |
| ppDDD | Agri 2 | Urban | 1.87 | 0.0619 | two.sided | z | None | Dunn |
| ppDDD | Agri 2 | Wood | 0.10 | 0.9176 | two.sided | z | None | Dunn |
| **ppDDD** | **Urban** | **Wood** | **2.54** | **0.0111** | **two.sided** | **z** | **None** | **Dunn** |
| opDDT | Agri 1 | Agri 2 | 0.81 | 0.4190 | two.sided | z | None | Dunn |
| **opDDT** | **Agri 1** | **Urban** | **3.13** | **0.0017** | **two.sided** | **z** | **None** | **Dunn** |
| opDDT | Agri 1 | Wood | 1.02 | 0.3061 | two.sided | z | None | Dunn |
| **opDDT** | **Agri 2** | **Urban** | **3.13** | **0.0017** | **two.sided** | **z** | **None** | **Dunn** |
| opDDT | Agri 2 | Wood | 1.59 | 0.1125 | two.sided | z | None | Dunn |
| **opDDT** | **Urban** | **Wood** | **2.18** | **0.0289** | **two.sided** | **z** | **None** | **Dunn** |
| ppDDT | Agri 1 | Agri 2 | 0.97 | 0.3322 | two.sided | z | None | Dunn |
| **ppDDT** | **Agri 1** | **Urban** | **2.41** | **0.0160** | **two.sided** | **z** | **None** | **Dunn** |
| ppDDT | Agri 1 | Wood | 0.24 | 0.8088 | two.sided | z | None | Dunn |
| **ppDDT** | **Agri 2** | **Urban** | **2.77** | **0.0056** | **two.sided** | **z** | **None** | **Dunn** |
| ppDDT | Agri 2 | Wood | 1.17 | 0.2408 | two.sided | z | None | Dunn |
| **ppDDT** | **Urban** | **Wood** | **2.27** | **0.0231** | **two.sided** | **z** | **None** | **Dunn** |
| Endosulfan | Agri 1 | Agri 2 | 0.70 | 0.4834 | two.sided | z | None | Dunn |
| **Endosulfan** | **Agri 1** | **Urban** | **2.53** | **0.0113** | **two.sided** | **z** | **None** | **Dunn** |
| Endosulfan | Agri 1 | Wood | 0.51 | 0.6130 | two.sided | z | None | Dunn |
| **Endosulfan** | **Agri 2** | **Urban** | **2.58** | **0.0099** | **two.sided** | **z** | **None** | **Dunn** |
| Endosulfan | Agri 2 | Wood | 1.08 | 0.2787 | two.sided | z | None | Dunn |
| **Endosulfan** | **Urban** | **Wood** | **1.99** | **0.0471** | **two.sided** | **z** | **None** | **Dunn** |
| EndosulfanI | Agri 1 | Agri 2 | 0.74 | 0.4572 | two.sided | z | None | Dunn |
| EndosulfanI | Agri 1 | Urban | 1.78 | 0.0755 | two.sided | z | None | Dunn |
| EndosulfanI | Agri 1 | Wood | 0.22 | 0.8221 | two.sided | z | None | Dunn |
| **EndosulfanI** | **Agri 2** | **Urban** | **2.07** | **0.0381** | **two.sided** | **z** | **None** | **Dunn** |
| EndosulfanI | Agri 2 | Wood | 0.91 | 0.3611 | two.sided | z | None | Dunn |
| EndosulfanI | Urban | Wood | 1.53 | 0.1248 | two.sided | z | None | Dunn |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| EndosulfanII | NA | NA | 1.46 | 0.4826 | NA | NA | NA | NA |
| HCH | Agri 1 | Agri 2 | 0.18 | 0.8557 | two.sided | z | None | Dunn |
| HCH | Agri 1 | Urban | 1.91 | 0.0567 | two.sided | z | None | Dunn |
| HCH | Agri 1 | Wood | 0.82 | 0.4129 | two.sided | z | None | Dunn |
| HCH | Agri 2 | Urban | 1.58 | 0.1138 | two.sided | z | None | Dunn |
| HCH | Agri 2 | Wood | 0.79 | 0.4274 | two.sided | z | None | Dunn |
| HCH | Urban | Wood | 1.11 | 0.2653 | two.sided | z | None | Dunn |
| alfa | Agri 1 | Agri 2 | 0.36 | 0.7202 | two.sided | z | None | Dunn |
| **alfa** | **Agri 1** | **Urban** | **2.78** | **0.0054** | **two.sided** | **z** | **None** | **Dunn** |
| alfa | Agri 1 | Wood | 1.36 | 0.1735 | two.sided | z | None | Dunn |
| **alfa** | **Agri 2** | **Urban** | **2.46** | **0.0138** | **two.sided** | **z** | **None** | **Dunn** |
| alfa | Agri 2 | Wood | 1.38 | 0.1687 | two.sided | z | None | Dunn |
| alfa | Urban | Wood | 1.55 | 0.1219 | two.sided | z | None | Dunn |
| beta | Agri 1 | Agri 2 | 1.66 | 0.0976 | two.sided | z | None | Dunn |
| beta | Agri 1 | Urban | 0.31 | 0.7540 | two.sided | z | None | Dunn |
| beta | Agri 1 | Wood | 0.69 | 0.4911 | two.sided | z | None | Dunn |
| beta | Agri 2 | Urban | 1.96 | 0.0503 | two.sided | z | None | Dunn |
| **beta** | **Agri 2** | **Wood** | **2.21** | **0.0272** | **two.sided** | **z** | **None** | **Dunn** |
| beta | Urban | Wood | 0.42 | 0.6748 | two.sided | z | None | Dunn |
| gamma | Agri 1 | Agri 2 | 0.87 | 0.3869 | two.sided | z | None | Dunn |
| **gamma** | **Agri 1** | **Urban** | **2.78** | **0.0055** | **two.sided** | **z** | **None** | **Dunn** |
| gamma | Agri 1 | Wood | 0.45 | 0.6550 | two.sided | z | None | Dunn |
| gamma | Agri 2 | Urban | 1.23 | 0.2169 | two.sided | z | None | Dunn |
| gamma | Agri 2 | Wood | 0.54 | 0.5910 | two.sided | z | None | Dunn |
| **gamma** | **Urban** | **Wood** | **2.46** | **0.0139** | **two.sided** | **z** | **None** | **Dunn** |
| HCB | Agri 1 | Agri 2 | 0.95 | 0.3423 | two.sided | z | None | Dunn |
| **HCB** | **Agri 1** | **Urban** | **2.33** | **0.0196** | **two.sided** | **z** | **None** | **Dunn** |
| HCB | Agri 1 | Wood | 1.81 | 0.0711 | two.sided | z | None | Dunn |
| **HCB** | **Agri 2** | **Urban** | **2.70** | **0.0070** | **two.sided** | **z** | **None** | **Dunn** |
| **HCB** | **Agri 2** | **Wood** | **2.31** | **0.0208** | **two.sided** | **z** | **None** | **Dunn** |
| HCB | Urban | Wood | 0.49 | 0.6228 | two.sided | z | None | Dunn |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| PeCB | Agri 1 | Agri 2 | 0.51 | 0.6135 | two.sided | z | None | Dunn |
| **PeCB** | **Agri 1** | **Urban** | **3.46** | **0.0005** | **two.sided** | **z** | **None** | **Dunn** |
| PeCB | Agri 1 | Wood | 1.44 | 0.1492 | two.sided | z | None | Dunn |
| **PeCB** | **Agri 2** | **Urban** | **3.06** | **0.0022** | **two.sided** | **z** | **None** | **Dunn** |
| PeCB | Agri 2 | Wood | 1.59 | 0.1125 | two.sided | z | None | Dunn |
| **PeCB** | **Urban** | **Wood** | **2.08** | **0.0379** | **two.sided** | **z** | **None** | **Dunn** |

**Table 4.8.** Dunn's Test for multiple comparisons (p<0.05). In red those that shows significant differences in concentrations (ng/g lw) among locations.

| Chemical | group1 | group2 | statistic | p.value | alternative | distribution | p.adjust.method | test |
|---|---|---|---|---|---|---|---|---|
| **OCP** | **Agri 1** | **Agri 2** | **3.03** | **0.0024** | **two.sided** | **z** | **None** | **Dunn** |
| OCP | Agri 1 | Urban | 1.73 | 0.0833 | two.sided | z | None | Dunn |
| **OCP** | **Agri 1** | **Wood** | **3.63** | **0.0003** | **two.sided** | **z** | **None** | **Dunn** |
| OCP | Agri 2 | Urban | 1.90 | 0.0578 | two.sided | z | None | Dunn |
| OCP | Agri 2 | Wood | 0.41 | 0.6788 | two.sided | z | None | Dunn |
| **OCP** | **Urban** | **Wood** | **2.13** | **0.0334** | **two.sided** | **z** | **None** | **Dunn** |
| **DDT** | **Agri 1** | **Agri 2** | **2.93** | **0.0034** | **two.sided** | **z** | **None** | **Dunn** |
| DDT | Agri 1 | Urban | 1.73 | 0.0833 | two.sided | z | None | Dunn |
| **DDT** | **Agri 1** | **Wood** | **3.68** | **0.0002** | **two.sided** | **z** | **None** | **Dunn** |
| DDT | Agri 2 | Urban | 1.79 | 0.0731 | two.sided | z | None | Dunn |
| DDT | Agri 2 | Wood | 0.28 | 0.7825 | two.sided | z | None | Dunn |
| **DDT** | **Urban** | **Wood** | **2.18** | **0.0295** | **two.sided** | **z** | **None** | **Dunn** |
| opDDE | Agri 1 | Agri 2 | 1.16 | 0.2461 | two.sided | z | None | Dunn |
| opDDE | Agri 1 | Urban | 0.40 | 0.6902 | two.sided | z | None | Dunn |
| opDDE | Agri 1 | Wood | 0.13 | 0.8959 | two.sided | z | None | Dunn |
| opDDE | Agri 2 | Urban | 1.43 | 0.1530 | two.sided | z | None | Dunn |
| opDDE | Agri 2 | Wood | 1.09 | 0.2751 | two.sided | z | None | Dunn |
| opDDE | Urban | Wood | 0.54 | 0.5914 | two.sided | z | None | Dunn |
| **ppDDE** | **Agri 1** | **Agri 2** | **2.93** | **0.0034** | **two.sided** | **z** | **None** | **Dunn** |
| ppDDE | Agri 1 | Urban | 1.73 | 0.0833 | two.sided | z | None | Dunn |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| **ppDDE** | **Agri 1** | **Wood** | **3.68** | **0.0002** | **two.sided** | **z** | **None** | **Dunn** |
| ppDDE | Agri 2 | Urban | 1.79 | 0.0731 | two.sided | z | None | Dunn |
| ppDDE | Agri 2 | Wood | 0.28 | 0.7825 | two.sided | z | None | Dunn |
| **ppDDE** | **Urban** | **Wood** | **2.18** | **0.0295** | **two.sided** | **z** | **None** | **Dunn** |
| opDDD | Agri 1 | Agri 2 | 0.35 | 0.7252 | two.sided | z | None | Dunn |
| opDDD | Agri 1 | Urban | 0.59 | 0.5522 | two.sided | z | None | Dunn |
| opDDD | Agri 1 | Wood | 0.31 | 0.7549 | two.sided | z | None | Dunn |
| opDDD | Agri 2 | Urban | 0.15 | 0.8781 | two.sided | z | None | Dunn |
| opDDD | Agri 2 | Wood | 0.13 | 0.8977 | two.sided | z | None | Dunn |
| opDDD | Urban | Wood | 0.35 | 0.7289 | two.sided | z | None | Dunn |
| ppDDD | Agri 1 | Agri 2 | 0.75 | 0.4548 | two.sided | z | None | Dunn |
| ppDDD | Agri 1 | Urban | 1.62 | 0.1042 | two.sided | z | None | Dunn |
| ppDDD | Agri 1 | Wood | 0.60 | 0.5515 | two.sided | z | None | Dunn |
| ppDDD | Agri 2 | Urban | 0.41 | 0.6843 | two.sided | z | None | Dunn |
| ppDDD | Agri 2 | Wood | 1.21 | 0.2272 | two.sided | z | None | Dunn |
| **ppDDD** | **Urban** | **Wood** | **2.36** | **0.0184** | **two.sided** | **z** | **None** | **Dunn** |
| opDDT | Agri 1 | Agri 2 | 1.01 | 0.3124 | two.sided | z | None | Dunn |
| opDDT | Agri 1 | Urban | 1.65 | 0.0990 | two.sided | z | None | Dunn |
| opDDT | Agri 1 | Wood | 0.00 | 1.0000 | two.sided | z | None | Dunn |
| **opDDT** | **Agri 2** | **Urban** | **2.26** | **0.0239** | **two.sided** | **z** | **None** | **Dunn** |
| opDDT | Agri 2 | Wood | 1.04 | 0.3006 | two.sided | z | None | Dunn |
| opDDT | Urban | Wood | 1.74 | 0.0825 | two.sided | z | None | Dunn |
| ppDDT | Agri 1 | Agri 2 | 0.51 | 0.6135 | two.sided | z | None | Dunn |
| ppDDT | Agri 1 | Urban | 1.86 | 0.0635 | two.sided | z | None | Dunn |
| ppDDT | Agri 1 | Wood | 0.47 | 0.6418 | two.sided | z | None | Dunn |
| ppDDT | Agri 2 | Urban | 1.88 | 0.0598 | two.sided | z | None | Dunn |
| ppDDT | Agri 2 | Wood | 0.17 | 0.8630 | two.sided | z | None | Dunn |
| **ppDDT** | **Urban** | **Wood** | **2.46** | **0.0139** | **two.sided** | **z** | **None** | **Dunn** |
| Endosulfan | Agri 1 | Agri 2 | 0.68 | 0.4967 | two.sided | z | None | Dunn |
| Endosulfan | Agri 1 | Urban | 0.29 | 0.7748 | two.sided | z | None | Dunn |
| Endosulfan | Agri 1 | Wood | 0.22 | 0.8221 | two.sided | z | None | Dunn |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| Endosulfan | Agri 2 | Urban | 0.92 | 0.3585 | two.sided | z | None | Dunn |
| Endosulfan | Agri 2 | Wood | 0.51 | 0.6102 | two.sided | z | None | Dunn |
| Endosulfan | Urban | Wood | 0.53 | 0.5968 | two.sided | z | None | Dunn |
| EndosulfanI | Agri 1 | Agri 2 | 0.40 | 0.6865 | two.sided | z | None | Dunn |
| EndosulfanI | Agri 1 | Urban | 1.16 | 0.2453 | two.sided | z | None | Dunn |
| EndosulfanI | Agri 1 | Wood | 0.84 | 0.3993 | two.sided | z | None | Dunn |
| EndosulfanI | Agri 2 | Urban | 0.43 | 0.6691 | two.sided | z | None | Dunn |
| EndosulfanI | Agri 2 | Wood | 0.23 | 0.8153 | two.sided | z | None | Dunn |
| EndosulfanI | Urban | Wood | 0.25 | 0.8015 | two.sided | z | None | Dunn |
| EndosulfanII | Agri 1 | Urban | 1.35 | 0.1763 | two.sided | z | None | Dunn |
| EndosulfanII | Agri 1 | Wood | 1.64 | 0.1017 | two.sided | z | None | Dunn |
| EndosulfanII | Urban | Wood | 0.76 | 0.4469 | two.sided | z | None | Dunn |
| HCH | Agri 1 | Agri 2 | 0.63 | 0.5311 | two.sided | z | None | Dunn |
| HCH | Agri 1 | Urban | 1.39 | 0.1634 | two.sided | z | None | Dunn |
| HCH | Agri 1 | Wood | 0.48 | 0.6285 | two.sided | z | None | Dunn |
| HCH | Agri 2 | Urban | 1.67 | 0.0946 | two.sided | z | None | Dunn |
| HCH | Agri 2 | Wood | 1.00 | 0.3170 | two.sided | z | None | Dunn |
| HCH | Urban | Wood | 0.94 | 0.3470 | two.sided | z | None | Dunn |
| alfa | Agri 1 | Agri 2 | 0.81 | 0.4202 | two.sided | z | None | Dunn |
| alfa | Agri 1 | Urban | 0.47 | 0.6356 | two.sided | z | None | Dunn |
| alfa | Agri 1 | Wood | 0.24 | 0.8125 | two.sided | z | None | Dunn |
| alfa | Agri 2 | Urban | 0.45 | 0.6543 | two.sided | z | None | Dunn |
| alfa | Agri 2 | Wood | 0.65 | 0.5157 | two.sided | z | None | Dunn |
| alfa | Urban | Wood | 0.26 | 0.7966 | two.sided | z | None | Dunn |
| beta | Agri 1 | Agri 2 | 1.88 | 0.0603 | two.sided | z | None | Dunn |
| beta | Agri 1 | Urban | 1.88 | 0.0600 | two.sided | z | None | Dunn |
| beta | Agri 1 | Wood | 0.08 | 0.9333 | two.sided | z | None | Dunn |
| beta | Agri 2 | Urban | 0.59 | 0.5570 | two.sided | z | None | Dunn |
| beta | Agri 2 | Wood | 1.86 | 0.0624 | two.sided | z | None | Dunn |
| beta | Urban | Wood | 1.89 | 0.0590 | two.sided | z | None | Dunn |
| gamma | Agri 1 | Agri 2 | 1.73 | 0.0836 | two.sided | z | None | Dunn |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| gamma | Agri 1 | Urban | 0.04 | 0.9659 | two.sided | z | None | Dunn |
| gamma | Agri 1 | Wood | 0.45 | 0.6550 | two.sided | z | None | Dunn |
| gamma | Agri 2 | Urban | 1.84 | 0.0664 | two.sided | z | None | Dunn |
| **gamma** | **Agri 2** | **Wood** | **2.15** | **0.0316** | **two.sided** | **z** | **None** | **Dunn** |
| gamma | Urban | Wood | 0.56 | 0.5772 | two.sided | z | None | Dunn |
| **HCB** | **Agri 1** | **Agri 2** | **2.06** | **0.0393** | **two.sided** | **z** | **None** | **Dunn** |
| HCB | Agri 1 | Urban | 1.05 | 0.2949 | two.sided | z | None | Dunn |
| HCB | Agri 1 | Wood | 0.55 | 0.5830 | two.sided | z | None | Dunn |
| HCB | Agri 2 | Urban | 1.39 | 0.1659 | two.sided | z | None | Dunn |
| **HCB** | **Agri 2** | **Wood** | **2.52** | **0.0118** | **two.sided** | **z** | **None** | **Dunn** |
| HCB | Urban | Wood | 1.70 | 0.0891 | two.sided | z | None | Dunn |
| PeCB | Agri 1 | Agri 2 | 0.30 | 0.7619 | two.sided | z | None | Dunn |
| PeCB | Agri 1 | Urban | 1.20 | 0.2317 | two.sided | z | None | Dunn |
| PeCB | Agri 1 | Wood | 1.12 | 0.2642 | two.sided | z | None | Dunn |
| PeCB | Agri 2 | Urban | 1.19 | 0.2342 | two.sided | z | None | Dunn |
| PeCB | Agri 2 | Wood | 1.14 | 0.2549 | two.sided | z | None | Dunn |
| PeCB | Urban | Wood | 0.04 | 0.9654 | two.sided | z | None | Dunn |

PCA was applied to reveal the presence of distinctive patterns within the distributions of the target chemicals. Prior to PCA, all data were log-transformed, adding 1 to avoid the logarithm of 0. No further normalization or scaling transformations were applied (Figure 4.9.).

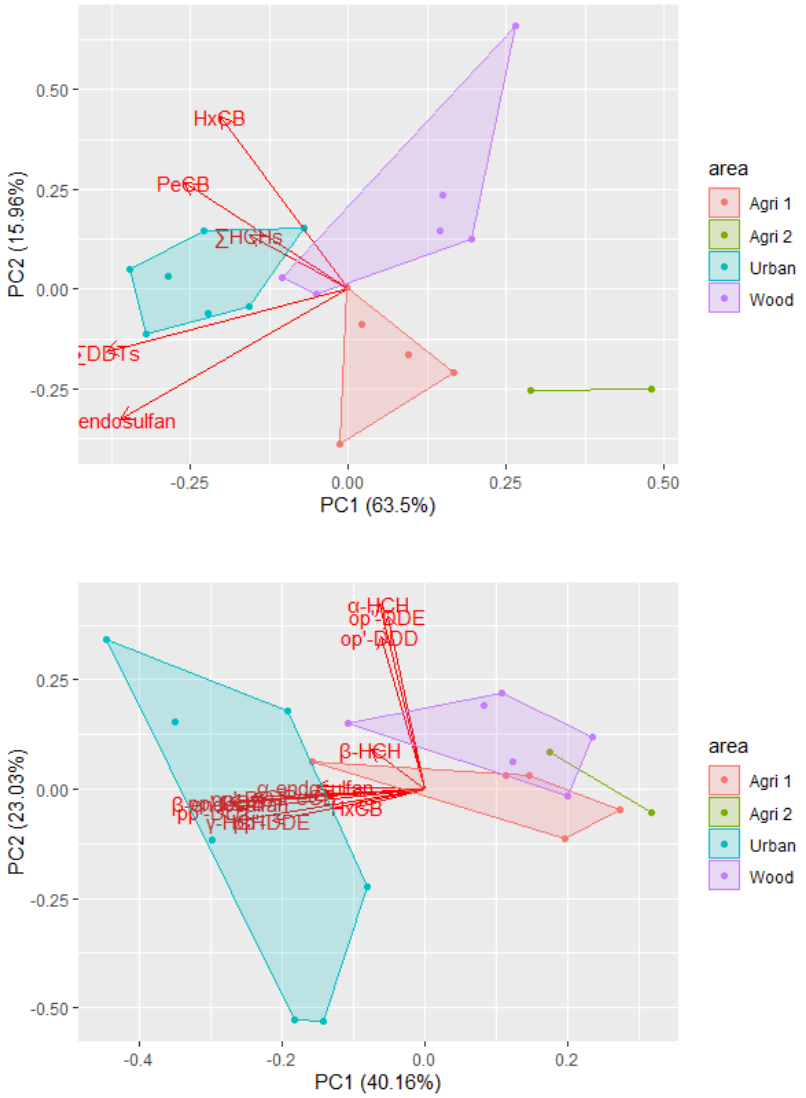


**Figure 4.9.** Plot of the results of principal component analysis (PCA) (first two components) conducted for target contaminants in ng/g ww. Samples are colored according to the locations from where they were collected.

### 4.3.2.3. PFAS levels in eggs

Levels of PFAS were evaluated in a total of 27 unhatched and abandoned eggs of great tit sampled in the study areas during 2021 and 2022. Concentrations data were expressed in ng/g ww (wet weight). As shown in Figure 4.10., the concentration of PFAS was higher in urban and agri areas than wood area. The agri 1 area also showed the highest levels of PFOS; however, these differences were not statistically significant. The highest levels of long-chain PFCA compounds were recorded in great tit's eggs from wood and urban areas. A statistically significant difference emerged between urban

and agri 1 areas for PFDoDA ($p<0.05$); levels of this compound were also significantly different between agri 1 and wood ($p<0.05$).

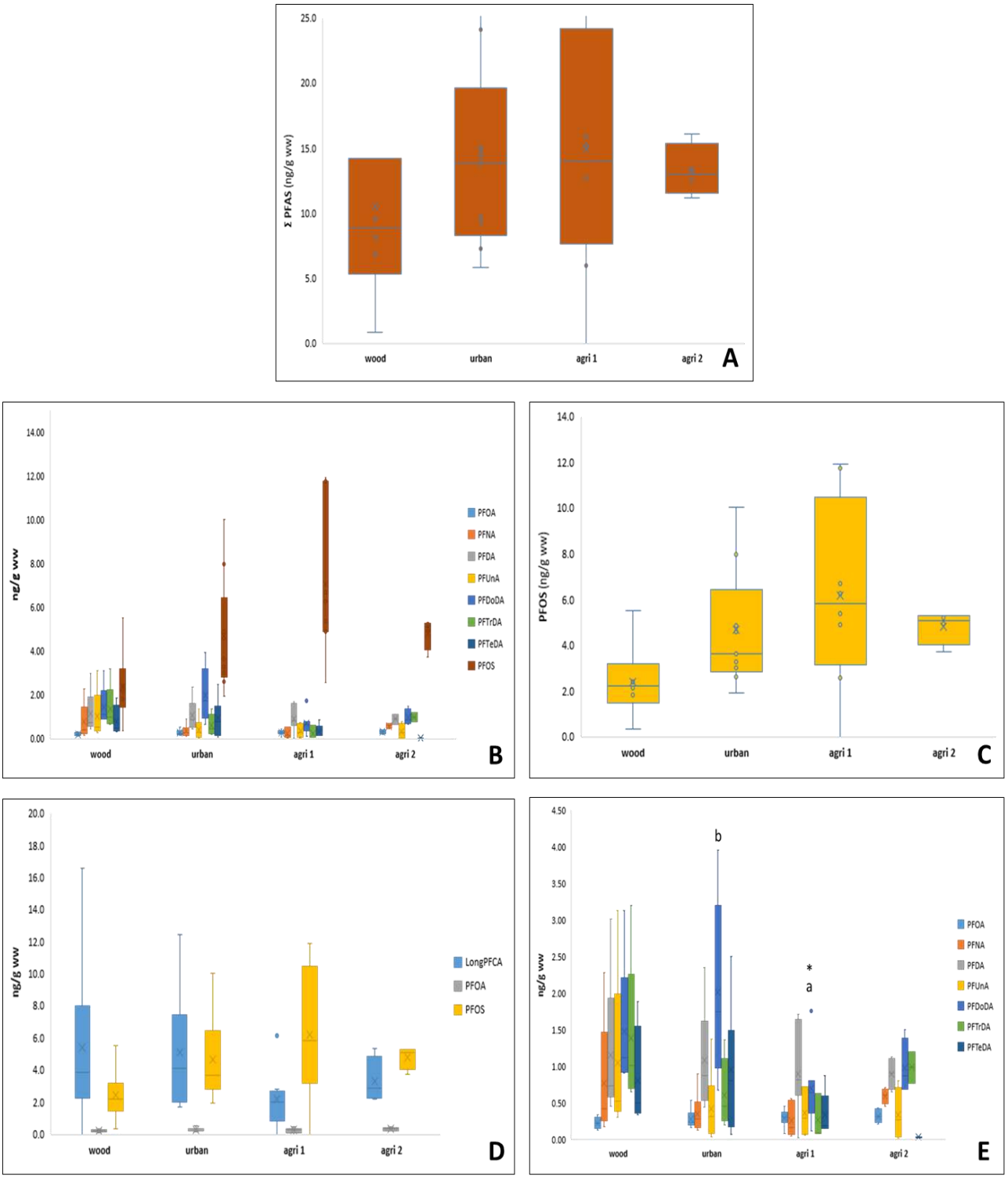


**Figure 4.10**. The concentration of PFAS compounds (expressed as ng/g ww) evaluated in great tit eggs sampled in the study areas during 2021 and 2022. The sum of PFAS (A), levels of the different PFAS class (B), detail on PFOS concentration (C), levels of long-chain PFCA (D), and concentration of PFAS excluding PFOS (E). * indicates a statistical significant difference from wood. Different letters indicate a statistically significant difference among areas ($p<0.05$).

The Veneto Region was interested by PFAS contamination case where surface and groundwaters were significantly impacted by perfluoroalkyl carboxylic acids (PFAA) discharges from a fluorochemical plant (Ferrario et al., 2022); however, it is important to underline that our study areas are not included in the known contaminated sites. PFAS are environmentally persistent and due to their limited environmental degradation are widely distributed around the globe in all environmental compartments, including air, water, soils and sediments, animals, plants, and in humans (Ferrario et al., 2022; Pitter et al., 2020). Food, drinking water and dust are the major sources of background exposure (Lorber and Egeghy, 2011; Fraser et al., 2013) and are often associated with toxic effects in organisms including humans. Indeed, has been reported that some PFAS, such as PFOA and PFAS, have endocrine-disruption properties, can increase levels of serum cholesterol, impaired thyroid function and induce insulin resistance, gestational diabetes, and pregnancy hypertension (Pitter et al., 2020).

PFOS was the most abundant compound (Figure 4.10.), and this is consistent with widespread contamination and the absence of close industrial sources of contamination for the other PFAS.

Overall, the recorded PFOS concentrations are lower than those found in great tit's eggs collected in an area without known PFAS point sources in Belgium (4.3-82.2 ng/g ww). However, PFCA levels in our great tit's eggs were similar to the levels found in the Belgium reference site (<LOQ-4.3 ng/g ww) (Groffen et al., 2017). Our PFOS values were also lower than those recorded in eggs of three insectivorous bird species (*Vanellus vanellus, Larus melanocephalus, Parus major)* sampled near a fluoro-chemical plant in Belgium (Lopez-Antia et al., 2017). The total PFAS concentrations recorded in our study areas were lower than those found in tree swallow eggs *(Tachycineta bicolor)* sampled in known contaminated sites in northeastern Michigan (Custer et al., 2019).

Perfluoroalkyl acids (PFAAs), such as PFOS and PFCAs, travel little in the atmosphere, while their uncharged precursors, such as perfluorosulfonamides and fluorotelomeric alcohols, can be airborne far from emission points and global distributed (Saini et al., 2023). Indeed, the wood area had lower PFOS concentrations probably because of its remoteness from sources. On the other hand, the highest levels of the different PFCA were found in urban and wood areas. PFCA levels recorded in the urban site can be due to proximity to the emission sources while the levels of PFCA in the wood site can be due to the capture of PFCA precursor by plants that determine their availability along the food chain (Wang et al., 2020). Actually, deciduous plant leaves effectively capture semi-volatile PFAS that adhere to surfaces of their leaves. When the leaves fall to the ground and decompose, they transfer captured PFAS into the soil (Wang et al., 2022). Through the food web, PFAS in soil can

transfer to invertebrates and to their avian predators. It has been shown that PFAS levels found in invertebrates, such as earthworms (Navarro et al., 2016) and isopods (Groffen et al., 2019a), can be a good proxy for those found in the tissues of their avian predators (Groffen et al., 2019a).
It is known that the atmospheric oxidation and microbial degradation in the soil of fluorotelomeric alcohols (n:2FTOH) mainly produce PFCA with an even number of carbon atoms (Wang et al., 2009). In agri 1, agri 2, and urban areas, there is an alternation between the concentration of PFCA with even or odd number of carbon atoms (prevalence of even ones) suggesting that these compounds originate from the degradation of precursors.
Nevertheless, in the eggs collected in the wood area there is not a prevalence of PFCA with an even number of carbon atoms, but it is quite evident an increase in concentration with the increasing perfluoroalkyl chain. In this case, adsorption on leaves and bioaccumulation in birds, which are favored by the length of the fluorinated alkyl chain, probably prevail dominating the PFCA contamination profile.

## 4.4. A multi-biomarker and complementary approach to evaluate the ecotoxicological status of great tit populations integrating chemical analysis, biomarker responses, and behavioural and breeding performances

### 4.4.1. Material and methods

#### 4.4.1.1. Study areas

The great tit specimens have been sampled in 4 different sites in the Veneto Region (Italy). Specifically, in the city of Padua and areas in its province as the Euganean Hills Regional Park, S. Antonio Village - Noventa Padovana, Cà di Mezzo Oasis, and the locality of Vaccarino. These sites are characterized by different anthropogenic impacts and can be distinguished in wooded, urban and agricultural areas.
The Euganean Hills (Figure 4.11.), occupying the south-western part of the province of Padua, are a group of hills of volcanic origin and their highest peak is about 601 m above sea level. In 1989, the area became a Regional Park, which stretches over 18.000 hectares (en.venezia.net). Euganean Hills are about 15 km far from Padua city centre. Euganean Hills are not included in the maximum area of exposure to the known PFAS contamination but are not far away from contaminated sites (arpa.veneto.it). Vegetation of this area consists mainly of *Robinia pseudoacacia*, *Castanea sativa* and *Quercus* trees (parcocollieuganei.com).

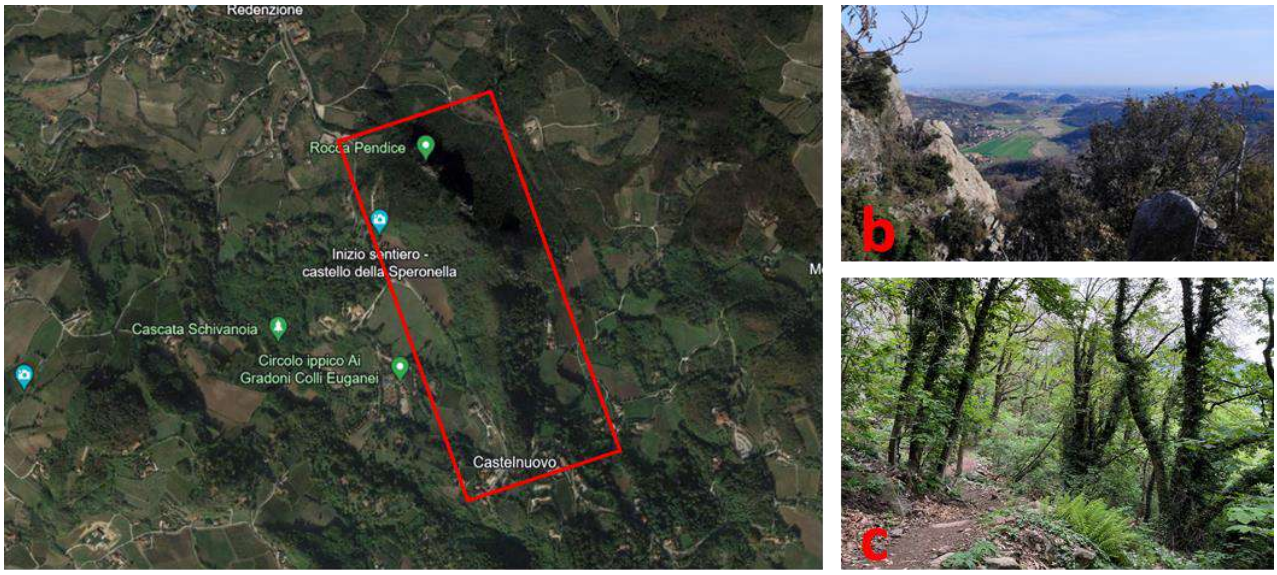


**Figure 4.11**. Sampling area of Euganean Hills Regional Park (a), details of the view from the hill (b) and of wood (c) where the nest boxes are located.

The urban area includes four sites that are all located in the centre of Padua city (Vallisneri, Esapolis and the Padua's botanical garden) and in its proximity (S. Antonio Village - Noventa Padovana). Vallisneri district is located between the Piovego canal and a high traffic street, close to the university complex. Villaggio Sant'Antonio is a recreation and reception centre for people with disabilities. This site's neighbourhood consists of an industrial area, mainly of storage of foods and products, but also some manufacturing industries, such as an industry which produces polyurethane and polyiso foam panels (Figure 4.12.).


**Figure 4.12.** Urban area: Vallisneri (a), Esapolis (b), Padua's botanical garden (c) and S. Antonio Village - Noventa Padovana (d) where the nest boxes are located.

The Cà di Mezzo Oasis, agricultural area (called agri 1 in this thesis), is an artificial phytodepuration site surrounded by intensive cultivation of wheat and corn. This site collects the wastewater of a canal which drains water from upstream farmland. It was created to remove nitrogen and phosphorus before waters are released into Venice lagoon. This area is also bordered by the Bacchiglione river, and it is nearby the Brenta river, known contaminated streams by compounds of industrial origin (Figure 4.13.).

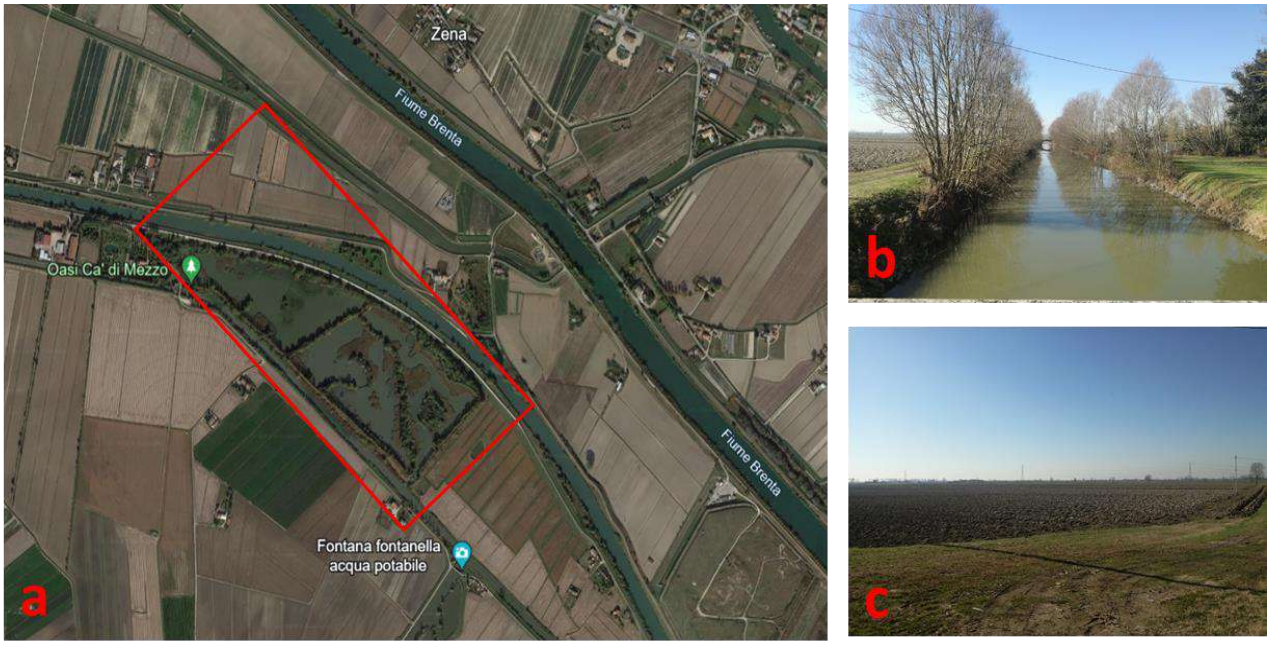


**Figure 4.13**. Sampling area of Cà di Mezzo Oasis (agri 1) (a), details of the canal that drains water from upstream farmland areas (b) and of the land cover surrounding the study area (c) where the nest boxes are located.

Vaccarino, the second agricultural area (called in this thesis agri 2), is 15 km away from Padua's city centre. This area is bordered by wheat crops and is not far away from a traffic street and from the Brenta river (Figure 4.14.).

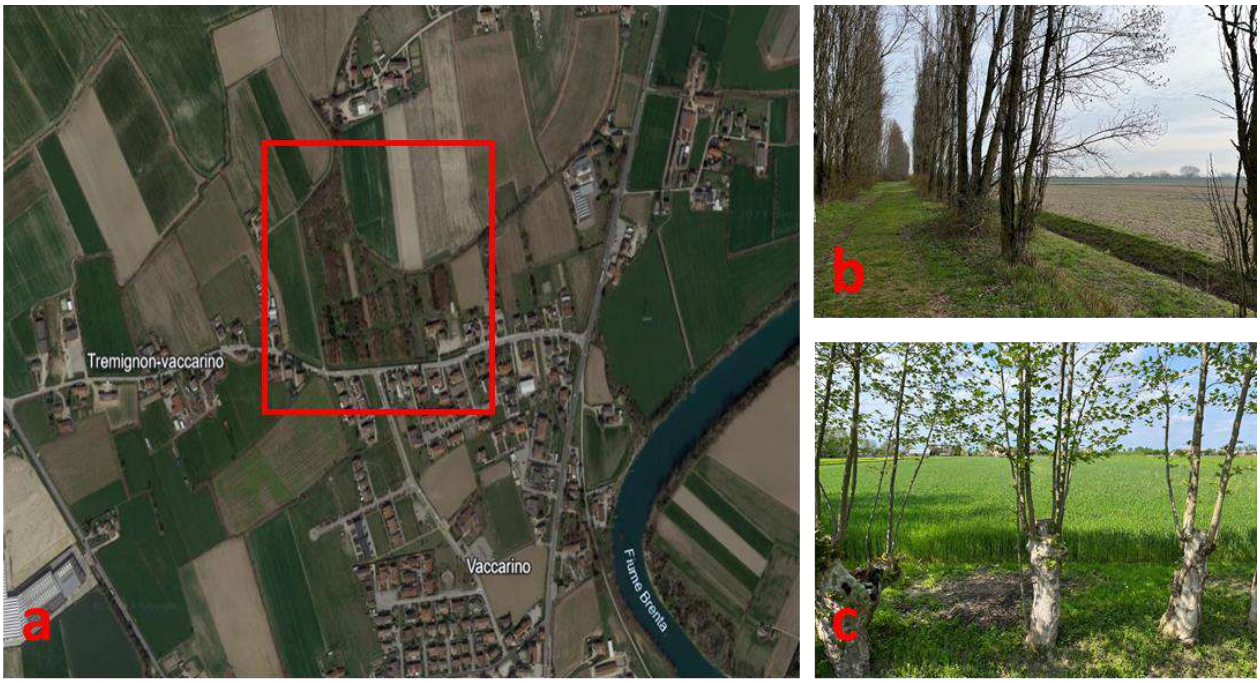


**Figure 4.14**. Sampling area of Vaccarino (agri 2) (a), details of the land cover surrounding the study area (b, c) where the nest boxes are located.

#### 4.4.1.2. Sampling plan

Currently, there are 180 nest boxes that have been installed in the past 6 years, 3 meters above the ground, on different trees species. The nest boxes are distributed as follows: 51 in the area of the Euganean Hills, 52 in the urban area, 61 in the locality of Vaccarino and 16 at the Cà di Mezzo Oasis. The monitoring campaigns were carried out for three consecutive years (2021, 2022 and 2023) during the great tit breeding season lasting from the middle of March to the end of May, when the last chicks fledge. During these campaigns, we monitored the reproductive performances, investigated the nestling's behavior, sampled biological material (blood, excreta, and feathers) from nestlings, and collected unhatched and abandoned eggs from nests.

**Monitoring of reproductive success**

The reproductive success monitoring was performed during great tit breeding season of 2021, 2022 and 2023.

**Behavioural investigation**

The behavioural tests were conducted in 2021, 2022 and 2023 on the great tit nestlings in which blood was subsequently sampled.

**Biological material sampling**

Blood (N= 198) and excreta (N= 143) samples were collected in 2021 and 2022 from great tit nestlings (43 nests). Nestlings aged 14-15 days were taken from the nest boxes and within a few minutes, behavioural tests and blood and excreta sampling was completed to minimise any stress conditions arising from handling. Blood sampling was performed using a needle and puncturing the brachial vein, then blood was collected by heparinized capillary tubes. Right after, two blood smears for each animal were prepared and left to dried at air. The remaining blood was transferred in vacutainer 500 µL (MICROMED®, Italy) containing lithium-heparin as an anticoagulant and processed. Blood samples were divided in several aliquots, an aliquot of whole blood was frozen, and an aliquot was mixed with a cryoconservation medium (RPMI:DMSO, 80:20). The remaining whole blood was centrifuged at 6000 g (Multispin 12, Argo LAB) for 10 minutes to obtain plasma. All the samples were stored in dry ice for the transport and stored at -80°C in the laboratory until analysis. The nestlings' weight was recorded with a field scale and the excreta produced by each animal was collected in tubes and stored at -20 °C. Feathers were sampled from a total of 159 nestlings (42 nests) during 2021 and 2022. The feathers were stored at room temperature in different paper bags until analysis.

#### 4.4.1.3. Procedure of reproductive success monitoring

Nest boxes were checked every 5 days to monitor the nest construction status in order to estimate its date end construction. The first egg laying date was recorded by checking the nests occupied by great tits every 3 days; after 10 days nest boxes were again checked to record clutch size data. Considering an incubation period of 13 days, the nest boxes were checked every day to determine the hatching date. Three days after hatching the brood sizes was recorded for hatching success calculation. Fledging success was quantified as percentage of brood size at three days.

#### 4.4.1.4. Behavioural tests

**Breath rate**

The breath rate (or respiratory frequency) test was applied on great tit nestlings following the method of Brommer & Kluen (2012). Each nestling was taken one at a time from a bag and placed with its back on the palm of the experimenter's hand and the head between the thumb and index finger (Figure 4.15.). The nestling was positioned 40 cm from the experimenter's face, and a one-minute video was recorded. Afterwards, each video was analyzed by counting the number of chest movements in 30 seconds. The measurement was repeated twice to assess repeatability.

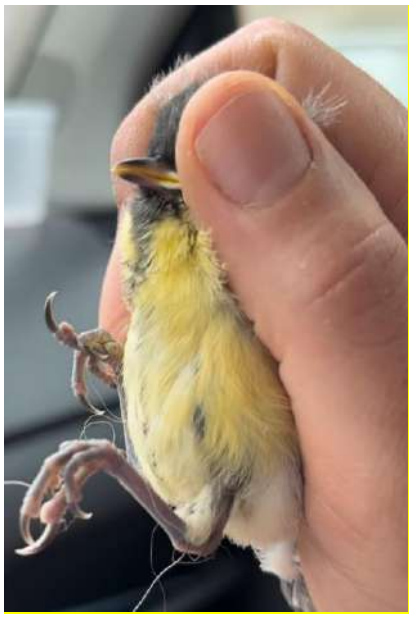

**Figure 4.15.** A *P. major* nestling subjected to the breath rate test.

### Agitation state

The agitation state was evaluated using the Corti et al. (2017) method. During the 60 seconds of the video in which the breath rate was measured, the number of nestling leg movements was also recorded.

### Tonic Immobility

The nestling was placed on its back in a shallow container on a horizontal base. The nestling was held with hands symmetrically positioned over the nestling and covering its body until it ceased struggling to escape. After removing the hands, the time until the nestlings moved to turn itself from a supine to a prone position was recorded, with a maximum duration set at 90 seconds (Figure 4.16.).

**Figure 4.16.** A *P. major* nestling during the tonic immobility test.

#### 4.4.1.5. Analytical methods

**Biomarkers**

A set of biomarkers was tested in the blood of great tits individuals sampled in 2021 and 2022 from all the study areas. We evaluated biomarkers of oxidative status (Total Antioxidant Status, TAS), porphyrin levels, immune system (complement system activity, respiratory burst and differential White Blood Cells, WBCs count), genotoxicity (comet assay and Erythrocyte Nuclear Abnormalities, ENA assays) and neurotoxicity (Butyrylcholinesterase, BChE and Carboxylesterase, CaE).

*TAS assay*

The TAS was evaluated by the use of a commercial kit (Antioxidant Assay Kit, Sigma, St. Louis, MO) through spectrophotometric method modified by Caliani et al. (2019). A stock solution of 1.5 mM of Trolox, diluted in assay buffer, was used to prepare the standard curve. Trolox concentrations aliquots and plasma samples (diluted 1:100 in assay buffer) were added to a 96-well plate in duplicate. Myoglobin and the chromogen ABTS were added to each well and the plate incubated at room temperature. Absorbance at 405 nm was measured using a multiplate reader (Multiskan Skyhigh Thermo Scientific). The TAS was expressed as mM of Trolox by linear regression of the standard curve.

*Porphyrins in excreta*

In the laboratory the excreta samples were freeze-dried and then transformed into powder through a pestling process (Figure 4.17.). Extraction of porphyrins from excreta was carried out using the method from Lockwood et al. (1985). From each sample, 50 mg of excreta were added to 1 mL of HCL 5N. After using the vortex mixer for 4 cycles of 10 seconds each, 3 mL of diethyl ether were added. Next, after another 3 cycles using the vortex mixer, 3 mL of distilled water diluted the obtained emulsion. Finally, following another 3 cycles of vortex, the samples were centrifuged at 2000 rpm for 5 minutes. Thanks to the centrifuge, three different layers were obtained, a supernatant of diethyl ether where carotenoid derivatives are located, a central layer of insoluble material and a watery sedimented portion where porphyrins are located. The porphyrins are in free-acids form. The quantitative determination of porphyrins was carried out by Grandchamp et al. (1980) method. Through this fluorimetric reading, it is possible to determine the concentration of uroporphyrins, coproporphyrins and protoporphyrins. The procedure is based on the use of three different pairs of excitation and emission wavelengths specific for each type of porphyrin (uroporphyrins 405-595 nm, coproporphyrins 400-595 nm, protoporphyrins 410-605 nm).

Recording the emissions of the fluorescence mixture at three different pairs of values, it is possible to obtain 3 linear functions representing concentrations of each porphyrin in the mixture compared to the standard curves previously analysed. Concentrations are expressed as $pmol^{-1}$ g dry weight of excreta.


**Figure 4.17**. Excreta sample of great tits before (a) and after (b) freeze-drying and pestling process.

*Respiratory burst*

The respiratory burst activity was measured with the NBT assay, following the method of Caliani et al. (2019), modified. Whole blood samples were added to a 96-well plate and incubated at 40 °C for 2 h. L-15 medium (100 µL ) supplemented with NBT (1 mg/mL) and PMA was added to each well and the plate was incubated at room temperature for 1 h. After incubation, the plate was fixed with 100% methanol, washed with 70% methanol and air-dried. KOH and then DMSO were added to each well in order dissolve the crystals of formazan blue deriving from the reduction of NBT by the oxyradicals. Absorbance was measured at 630 nm using a multiplate reader (Multiskan Skyhigh Thermo Scientific) using KOH/DMSO as white. The respiratory burst activity was expressed as a reduction of NBT.

*Differential white blood cells (WBC) count and H/L ratio*

Dried blood smears were stained with Diff-Quick stain and two hundred leukocytes were counted using an optical immersion microscope (Olympus BX41) at 100x magnification. Leukocytes were classified as heterophils, eosinophils, basophils, lymphocytes and monocytes according to the cellular morphology for birds described by Campbell (1995) (Figure 4.18).

Results are expressed as number of each leucocyte category per 200 leukocytes counted. The number of heterophils and lymphocytes counted in each animal, was used to calculate the H/L ratio, an index of physiological stress.

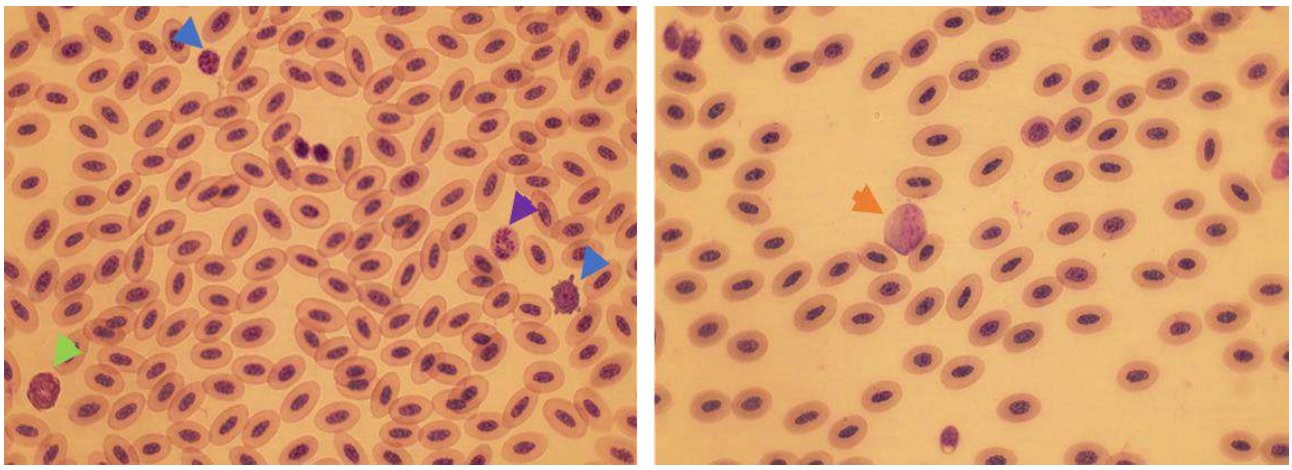

**Figure 4.18**. Leucocytes of *Parus major*: heterophil (green arrow), eosinophil (purple arrow), lymphocytes (blue arrows) and monocyte (orange arrow).

*Complement system activity*

Plasma complement activity was determined by the spectrophotometric method of sheep red blood cells (SRBCs) hemolysis. Whole blood of healthy Merino sheep (Ovis aries) was centrifuged to obtain fresh SRBCs. The SRBCs were washed and diluted with phosphate-buffered. Plasma samples were t incubated with 2% SRBC for 30 min at 37 °C. Afterwards, samples were centrifuged (2500× g for 5 min) and supernatant was used in microplate reader at 540 nm (Multiskan Skyhigh Thermo Scientific). A positive control was prepared adding Triton X-100 to 1% SRBC and homogenizing it until complete hemolysis; it was centrifuged and the optical density (O.D.) measured (considered 100% hemolysis). The results were expressed as mean % hemolysis.

*Comet assay*

Erythrocytes were processed for the comet assay according to Caliani et al. (2014) with some modifications. Blood was diluted in PBS, embedded within agarose gel and layered on two slides per sample, pre-dipped in 0.1% normal melting agarose. The slides were immersed into a freshly made lysis solution for at least 1 h at 4 °C in the dark. The slides were then placed on a horizontal electrophoresis tray previously filled with freshly prepared cold alkaline buffer and left for 15 min to allow DNA unwinding. Electrophoresis was performed at 16 V and 250 mA for 10 min. Slides were then neutralized and stained with SYBR® in TE buffer. To prevent DNA damage by photolysis, all

phases were conducted under yellow light or in the dark. A total of 100 cells per sample were examined under the epifluorescence microscope (Olympus BX41) at 40× magnification (Figure 4.19). The amount of DNA damage was evaluated as the DNA tail % using an image analyzer (6.0 Software, Kinetic Imaging Ltd.).

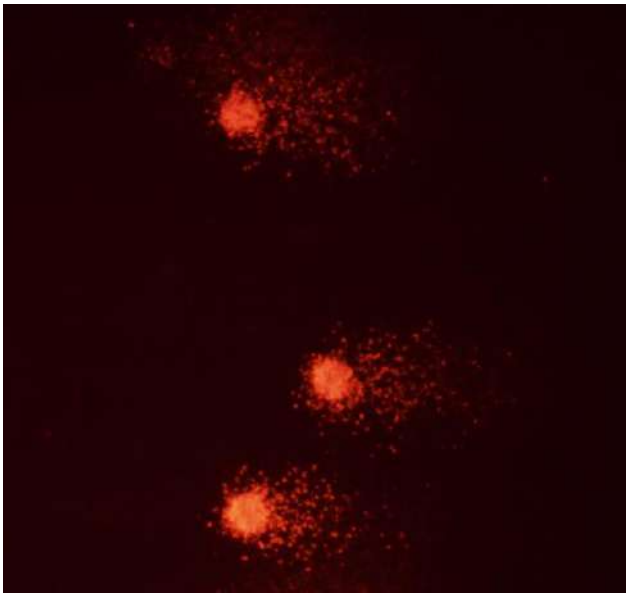

**Figure 4.19.** Blood cells of *Parus major* obtained by comet assay.

*ENA assay*

Quantification of ENAs was conducted in the same slides used for the differential leukocyte count. A total of 2000 mature erythrocytes per sample was analysed and quantified at 100x magnification using an optical immersion microscope (Olympus BX41). We classified the nuclear alterations as follow: bud, segmented, notched, kidney and micronucleus (Figure 4.20).

Results were expressed as number of each anomaly per 2000 erythrocytes and as a total number of abnormalities per 2000 cells.

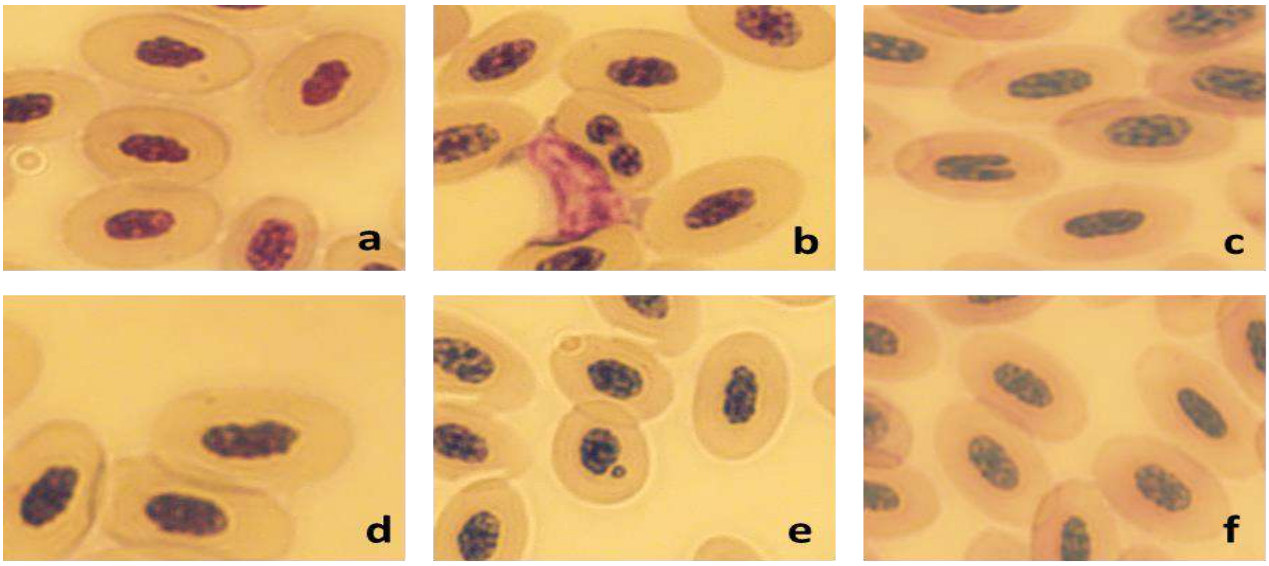


**Figure 4.20.** Nuclear abnormalities in erythrocytes of *Parus major*: bud (a), segmented, (b) notched (c), kidney (d) micronucleus (e) and normal (f).

*BChE activity*

Butyrylcholinesterase (BChE) activity was evaluated in plasma samples using a spectrophotometric test. The reaction assay mixture contained: TRIS-HCl buffer, CaCl2, DTNB, BTCI and plasma. Samples were run in double using a 96-well plate. Absorbance was measured at 410 nm with a microplate reader (Multiskan Skyhigh Thermo Scientific) at 40°C every 30 s for 5 minutes. The activity was expressed as µmol/min/ml plasma.

*CaE activity*

Carboxylesterase activity was evaluated by a spectrophotometric method. To 96-wells plate we added the reaction assay mixture, which contained: phosphate buffer, the substrate p-nitrophenyl butyrate and plasma. Samples and white were run in double in a microplate reader (Multiskan Skyhigh Thermo Scientific) measuring the absorbance at 405 nm at 40 °C every 30 s for 5 minutes. The activity was expressed as nmol/min/ml plasma.

These biomarkers were also assessed on common kestrel individuals, for more details on protocols refer to chapter 3 (section 3.5.).

#### 4.4.1.6. Statistical analysis

All the statistical analyses were performed using R software (version 4.1.3, R Foundation for Statistical Computing). To study the effect of different study areas with different anthropogenic impacts on biological responses of great tit individuals, univariate mixed models were applied (one

model for each biomarker). In particular, the single biomarker was always included as a dependent variable, while “area” was included as a fixed factor (4 levels: wood, urban, Agri 1 and Agri 2). Moreover, “Year” (2 levels: 2021 and 2022), “body mass” and “sampling day” were included as fixed factor and covariates, respectively, to correct for the possible effect of sampling year, body condition and breeding period. “Nest identity” was also included as random factors in all models.

First, regarding oxidative status biomarkers we performed a linear mixed model (LMM, function “lmer()”, package “lmerTest”) with “TAS” as dependent variables, while with regard to porphyrins, a series of LMMs was applied to “coproporphyrins”, “uroporphyrins”, “protoporphyrins” and “total porphyrins”. A log-transformation was applied for coproporphyrins, while a square-root transformation was applied for uroporphyrins and protoporphyrins. Second, regarding immunotossicity biomarkers, LMMs were used for “complement system activity”, “respiratory burst” and “H/L ratio”. Before running the models, a log-transformation was applied to all three dependent variables to meet the assumption of normality of the residuals. A series of generalised linear mixed models (GLMMs, function “glmer()”, package “lmerTest”) was instead applied for count variables such as “heterophyles”, “eosinophils”, “lymphocytes” and “monocytes”. In this case, a Poisson error distribution was considered, and an observation-level random effect was include in “eosinophils”, “lymphocytes” and “monocytes” models to control for overdispersion (Harrison, 2014; Pearson’s Chi-squared test: all $p < 0.05$). Third, regarding genotoxicity biomarkers, we fitted a LMM for the “comet assay” biomarker. In this case, a square-root transformation was applied to achieve normality of the residuals. A GLMM with a Poisson error distribution, was instead fitted for “total ENAs”, “buds”, “segmented”, “kidney”, “notched” nuclei, and for “micronucleus”. For all these Poisson models, we also included an observation-level random effect to control for overdispersion (Harrison, 2014), which was confirmed by a Pearson’s Chi-squared test (all $p < 0.05$). Finally, with regard to neurotoxicity biomarkers, we performed two LMMs with “BChE” and “CaE” as dependent variables. In this case, in order to achieve residual normality, a log-transformation was applied for neurotoxicity biomarkers (BChE, CaE) only.

For behavioural tests and reproductive success, the same statistical analyses used for the biomarkers was applied.

### 4.4.2. Results and discussion

#### 4.4.2.1. Biomarker responses

We evaluated biomarkers of oxidative status (Total Antioxidant Status, TAS), porphyrins levels, immune system (complement system activity, respiratory burst and differential White Blood Cells (WBCs)count), genotoxicity (comet assay and Erythrocyte Nuclear Abnormalities (ENA) assay) and neurotoxicity (Butyrylcholinesterase (BChE) and Carboxylesterase, (CaE)) in great tit individuals, sampled in 2021 and 2022, from areas with different anthropic impacts. The study areas were distinguished in a wood, an urban, and two agricultural areas, Oasi Cà di Mezzo and Vaccarino, namely agri 1 and agri 2, respectively. We investigated the potential effects of sampling years, sampling day and body mass on each biomarker.

Mean, standard deviation, min and max values of biomarkers (Table 4.9) and the outcomes of linear mixed models divided per biomarker with similar biological responses (Tables 4.10, 4.11, 4.12, 4.13, 4.14) are summarized below.

**Table 4.9**. Mean ± SD and range values of biomarkers with relative sample size (N) of great tit nestlings sampled in wood, urban, agri 1 and agri 2 study areas during 2021 and 2022.

| Biomarker | Wood | | | Urban | | | Agri 1 | | | Agri 2 | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Mean ± SD | min-max | N | Mean ± SD | min-max | N | Mean ± SD | min-max | N | Mean ± SD | min-max | N |
| **TAS** mM Trolox | 15.12±11.78 | 0.00-36.17 | 36 | 12.06±13.28 | 0.00-51.39 | 25 | 19.60±9.67 | 0.00-35.21 | 17 | 16.82±11.01 | 0.00-43.24 | 36 |
| *Coproporphyrins* pmol/g d.w. | 2379.44±1175.04 | 349.15-5220.18 | 49 | 2334.36±872.07 | 1240.87-4625.14 | 23 | 1680.92±752.79 | 60.63-3621.20 | 31 | 2166.73±868.27 | 863.27-4583.47 | 40 |
| *Uroporphyrins* pmol/g d.w. | 2446.21±1239.48 | 507.43 - 5734.51 | 47 | 2338.82±1037.51 | 632.88-5103.83 | 23 | 1931.35±1038.63 | 304.42-4289.34 | 31 | 1865.19±858.12 | 410.24-4175.46 | 40 |
| *Protoporphyrins* pmol/g d.w. | 6955.10±3578.61 | 1250.00 - 15943.20 | 49 | 6107.61±3076.49 | 63.12-13175.00 | 23 | 5516.83±2509.68 | 1001.26-13793.21 | 31 | 5036.77±2093.69 | 1852.00-10254.55 | 40 |
| **Total porphyrins** pmol/g d.w. | 11712.83±5601.68 | 2196.15 - 26077.06 | 49 | 10866.97±4256.39 | 5050.47-19605.46 | 23 | 9157.41±3665.00 | 1366.32-18200.92 | 31 | 9075.54±3224.52 | 3511.27-17388.18 | 40 |
| **Complement system activity** Haemolysis % | 40.61±29.02 | 16.90 - 158.28 | 38 | 67.61±82.15 | 10.83-295.20 | 14 | 50.39±49.19 | 10.58-184.42 | 23 | 80.25±64.93 | 21.75-312.53 | 28 |
| **Respiratory Burst** O.D. 630 nm | 0.33±0.35 | 0.05-1.44 | 43 | 0.18±0.14 | 0.05-0.57 | 26 | 0.32±0.42 | 0.06-1.56 | 21 | 0.31±0.59 | 0.07-2.79 | 29 |
| *Heterophils* N/200 leukocytes | 51.18±14.47 | 20-90 | 58 | 83.40±29.40 | 29-144 | 47 | 59.83±25.24 | 15-130 | 38 | 64.68±27.48 | 8-155 | 55 |
| *Eosinophils* N/200 leukocytes | 3.82±4.03 | 0-19 | 58 | 4.48±3.11 | 0-15 | 47 | 2.68±2.86 | 0-10 | 38 | 4.32±4.38 | 0-22 | 55 |
| *Lymphocytes* N/200 leukocytes | 140.16±17.12 | 83-176 | 58 | 108.26±29.22 | 47-165 | 47 | 134.54± 26.48 | 64-182 | 38 | 125.48±27.41 | 44-174 | 55 |
| *Monocytes* N/200 leukocytes | 4.71±5.21 | 0-21 | 58 | 3.840±2.902 | 0-11 | 47 | 2.88±2.78 | 0-9 | 38 | 3.59±4.46 | 0-27 | 55 |
| **H:L ratio** | 0.38±0.17 | 0.11-1.08 | 58 | 0.935±0.664 | 0.18-3.06 | 47 | 0.51±0.38 | 0.08-2.03 | 38 | 0.75±1.21 | 0.08-3.52 | 55 |
| **Comet assay** DNA tail % | 12.58±4.65 | 5.24-26.62 | 66 | 14.165±6.383 | 6.02-27.88 | 38 | 14.51±7.24 | 6.53-32.43 | 42 | 13.15±4.39 | 7.17-28.44 | 52 |

| | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **ENAs assay** Σ N abnormalities /2000 cells | 42.167 ± 19.468 | 16 - 122 | 61 | 63.566 ± 28.867 | 23 - 174 | 44 | 56.133 ± 23.183 | 21 - 112 | 38 | 61.045 ± 27.659 | 18 - 162 | 55 |
| *Bud* N/2000 cells | 28.139 ± 14.317 | 10 -100 | 61 | 47.151 ± 23.676 | 15 -125 | 44 | 40.400 ±17.754 | 6 - 82 | 38 | 43.924 ± 21.459 | 12 - 103 | 55 |
| *Segmented* N/2000 cells | 4.500 ± 3.933 | 0 -17 | 61 | 3.302 ± 3.308 | 0 - 19 | 44 | 3.800 ± 2.989 | 0 - 12 | 38 | 3.545 ± 3.259 | 0 - 12 | 55 |
| *Notched* N/2000 cells | 2.222 ± 2.272 | 0 - 9 | 61 | 4.868 ±4.545 | 0 - 22 | 44 | 3.356 ± 3.185 | 0 - 12 | 38 | 3.439 ± 2.899 | 0 - 13 | 55 |
| *Kidney* N/2000 cells | 7.069 ± 3.817 | 2 - 15 | 61 | 7.642 ± 4.537 | 0 - 22 | 44 | 8.156 ± 5.022 | 1 - 21 | 38 | 9.833 ± 6.644 | 0 - 35 | 55 |
| *MN* N/2000 cells | 0.236 ± 0.569 | 0 - 3 | 61 | 0.604 ± 1.132 | 0 - 6 | 44 | 0.422 ± 0.723 | 0 - 3 | 38 | 0.303 ± 0.744 | 0 - 4 | 55 |
| **BChE activity** µmol/min/mL | 0.764 ± 0.478 | 0.002 - 2.137 | 36 | 0.657 ± 0.389 | 0.114 - 1.439 | 21 | 0.515 ± 0.235 | 0.163 - 0.918 | 19 | 0.769 ± 0.491 | 0.131 - 2.391 | 32 |
| **CaE activity** nmol/min/mL | 507.118 ± 356.653 | 45.334 - 1715.546 | 39 | 431.470 ± 276.947 | 71.238 - 1160.148 | 26 | 352.993 ± 161.446 | 139.894 - 853.407 | 27 | 342.516 ± 187.096 | 116.622 - 861.000 | 28 |

**Table 4.10**. Outcomes of linear mixed models used to evaluate the effects of each study area on oxidative status in great tit (*Parus major*) nestlings sampled in 2021 and 2022. *p< 0.05, indicates a significant difference.

| Variable | Factor | Reference Level | Level | Coefficient Estimate ± SE | *t* | *p* |
|---|---|---|---|---|---|---|
| **TAS** | *Area* | Wood | Urban | -5.043 ± 3.700 | -1.363 | 0.184 |
| | | Wood | Agri 1 | 7.956 ± 4.112 | 1.935 | 0.064 |
| | | Wood | Agri 2 | 2.081 ± 3.419 | 0.609 | 0.549 |
| | | Urban | Agri 1 | 13.000 ± 4.084 | 3.183 | 0.003* |
| | | Urban | Agri 2 | 7.124 ± 3.683 | 1.934 | 0.064 |
| | *Sampling year* | 2021 | 2022 | 10.600 ± 2.507 | 4.229 | 0.0003* |
| | *Sampling day* | | | -0.578 ± 0.327 | -1.768 | 0.089 |
| | *Body mass* | | | -2.435 ± 0.735 | -3.313 | 0.002* |

**Table 4.11**. Outcomes of linear mixed models used to evaluate the effects of each area on porphyrin levels in great tit (*Parus major*) nestlings sampled in 2021 and 2022. *p< 0.05, indicates a significant difference.

| Variable | Factor | Reference Level | Level | Coefficient Estimate ± SE | *t* | *p* |
|---|---|---|---|---|---|---|
| **Coproporphyrins** | *Area* | Wood | Urban | -0.0224 ± 0.186 | -0.12 | 0.905 |
| | | Wood | Agri 1 | -0.438 ± 0.185 | -2.367 | 0.025* |
| | | Wood | Agri 2 | -0.130 ± 0.160 | -0.812 | 0.423 |
| | *Sampling year* | 2021 | 2022 | -0.318 ± 0.125 | -2.545 | 0.015* |
| | *Sampling day* | | | 0.022 ± 0.013 | 1.669 | 0.105 |
| | *Body mass* | | | 0.032 ± 0.034 | 0.922 | 0.359 |
| **Uroporphyrins** | *Area* | Wood | Urban | -2.170 ± 4.397 | -0.493 | 0.624 |
| | | Wood | Agri 1 | -8.614 ± 4.507 | -1.911 | 0.065 |
| | | Wood | Agri 2 | -7.709 ± 3.886 | -1.984 | 0.056 |
| | *Sampling year* | 2021 | 2022 | -0.337 ± 2.999 | -0.112 | 0.911 |
| | *Sampling day* | | | 0.404 ± 0.315 | 1.282 | 0.208 |
| | *Body mass* | | | 0.062 ± 0.771 | 0.081 | 0.936 |
| **Protoporphyrins** | *Area* | Wood | Urban | -10.144 ± 7.076 | -1.434 | 0.159 |
| | | Wood | Agri 1 | -10.706 ± 7.160 | -1.495 | 0.148 |
| | | Wood | Agri 2 | -13.209 ± 6.181 | -2.137 | 0.042* |
| | *Sampling year* | 2021 | 2022 | -7.260 ± 4.798 | -1.513 | 0.141 |
| | *Sampling day* | | | 0.521 ± 0.503 | 1.036 | 0.309 |
| | *Body mass* | | | 1.410 ± 1.268 | 1.112 | 0.267 |
| **Total porphyrins** | *Area* | Wood | Urban | -1743.28 ± 1603.46 | -1.087 | 0.283 |
| | | Wood | Agri 1 | -3416.44 ± 1601.93 | -2.133 | 0.042* |
| | | Wood | Agri 2 | -3407.18 ± 1384.59 | -2.461 | 0.019* |
| | *Sampling year* | 2021 | 2022 | -1386.00 ± 1079.18 | -1.284 | 0.208 |
| | *Sampling day* | | | 157.56 ± 112.83 | 1.397 | 0.172 |
| | *Body mass* | | | 215.72 ± 293.64 | 0.735 | 0.464 |

**Table 4.12.** Outcomes of linear mixed models used to examine the effects of each area on immune system in great tit (*Parus major*) nestlings sampled in 2021 and 2022. *p< 0.05, indicates significant difference.

| Variable | Factor | Reference Level | Level | Coefficient Estimate ± SE | *t/z* | *p* |
|---|---|---|---|---|---|---|
| **Complement system activity** | *Area* | Wood | Urban | 0.411 ± 0.228 | 1.802 | 0.075 |
| | | Wood | Agri 1 | 0.120 ± 0.205 | 0.585 | 0.560 |
| | | Wood | Agri 2 | 0.662 ± 0.193 | 3.419 | 0.0009* |
| | | Agri 1 | Urban | 0.291 ± 0.245 | 1.188 | 0.238 |
| | | Agri 1 | Agri 2 | 0.542 ± 0.194 | 2.797 | 0.006* |
| | *Sampling year* | 2021 | 2022 | 0.539 ± 0.196 | 2.755 | 0.007* |
| | *Sampling day* | | | 0.008 ± 0.019 | 0.441 | 0.661 |
| | *Body mass* | | | 0.114 ± 0.049 | 2.326 | 0.022* |
| **Respiratory burst** | *Area* | Wood | Urban | -0.300 ± 0.252 | -1.192 | 0.236 |
| | | Wood | Agri 1 | -0.418 ± 0.242 | -1.729 | 0.087 |
| | | Wood | Agri 2 | -0.385 ± 0.213 | -1.811 | 0.073 |
| | *Sampling year* | 2021 | 2022 | 0.841 ± 0.168 | 4.999 | <0.0001* |
| | *Sampling day* | | | 0.034 ± 0.021 | 1.667 | 0.098 |
| | *Body mass* | | | -0.011 ± 0.056 | -0.192 | 0.848 |
| **Differential WBCs count** | *Area* | Wood | Urban | 0.310 ± 0.127 | 2.436 | 0.015* |
| ***Heterophils*** | | Wood | Agri 1 | 0.072 ± 0.149 | 0.486 | 0.622 |
| | | Wood | Agri 2 | 0.303 ± 0.125 | 2.415 | 0.016* |
| | *Sampling year* | 2021 | 2022 | -0.070 ± 0.091 | -0.769 | 0.442 |
| | *Sampling day* | | | -0.024 ± 0.051 | -0.477 | 0.634 |
| | *Body mass* | | | -0.100 ± 0.009 | -10.906 | <0.0001* |
| **Differential WBCs count** | *Area* | Wood | Urban | 0.275 ± 0.197 | 1.395 | 0.163 |
| ***Eosinophils*** | | Wood | Agri 1 | -0.278 ± 0.226 | -1.228 | 0.219 |
| | | Wood | Agri 2 | 0.173 ± 0.190 | 0.914 | 0.361 |
| | | Urban | Agri 1 | -0.552 ± 0.224 | -2.463 | 0.014* |
| | | Urban | Agri 2 | -0.101 ± 0.193 | -0.524 | 0.601 |
| | *Sampling year* | 2021 | 2022 | 0.241 ± 0.141 | 1.711 | 0.087 |
| | *Sampling day* | | | -0.117 ± 0.080 | -1.474 | 0.141 |
| | *Body mass* | | | -0.074 ± 0.080 | -0.922 | 0.357 |

| | | | | | | |
|---|---|---|---|---|---|---|
| **Differential WBCs count** | *Area* | Wood | Urban | -0.176 ± 0.055 | -3.222 | 0.0013* |
| ***Lymphocytes*** | | Wood | Agri 1 | 0.002 ± 0.061 | 0.028 | 0.978 |
| | | Wood | Agri 2 | -0.094 ± 0.053 | -1.788 | 0.074 |
| | | Urban | Agri 1 | 0.178 ± 0.061 | 2.91 | 0.004* |
| | | Urban | Agri 2 | 0.082 ± 0.055 | 1.495 | 0.135 |
| | *Sampling year* | 2021 | 2022 | 0.055 ± 0.038 | 1.432 | 0.152 |
| | *Sampling day* | | | -0.001 ± 0.022 | -0.046 | 0.963 |
| | *Body mass* | | | 0.092 ± 0.018 | 5.079 | <0.0001* |
| **Differential WBCs count** | *Area* | Wood | Urban | -0.035 ± 0.200 | -0.173 | 0.862 |
| ***Monocytes*** | | Wood | Agri 1 | -0.424 ± 0.229 | -1.85 | 0.064 |
| | | Wood | Agri 2 | -0.128 ± 0.190 | -0.671 | 0.502 |
| | *Sampling year* | 2021 | 2022 | -1.011 ± 0.139 | -7.298 | <0.0001* |
| | *Sampling day* | | | 0.090 ± 0.078 | 1.16 | 0.246 |
| | *Body mass* | | | 0.170 ± 0.078 | 2.174 | 0.030* |
| **H/L ratio** | *Area* | Wood | Urban | 0.445 ± 0.182 | 2.441 | 0.019* |
| | | Wood | Agri 1 | 0.026 ± 0.203 | 0.128 | 0.899 |
| | | Wood | Agri 2 | 0.283 ± 0.175 | 1.62 | 0.114 |
| | | Urban | Agri 1 | -0.419 ± 0.203 | -2.066 | 0.046* |
| | | Urban | Agri 2 | -0.162 ± 0.182 | -0.891 | 0.378 |
| | *Sampling year* | 2021 | 2022 | -0.170 ± 0.127 | -1.339 | 0.189 |
| | *Sampling day* | | | -0.006 ± 0.014 | -0.432 | 0.664 |
| | *Body mass* | | | -0.161 ± 0.033 | -4.859 | <0.0001* |

**Table 4.13**. Outcomes of linear mixed models used to examine effects of each area on genotoxic parameters in great tit (*Parus major*) nestlings sampled in 2021 and 2022. *p< 0.05, indicates significant difference.

| Variable | Factor | Reference Level | Level | Coefficient Estimate ± SE | *t/z* | *p* |
|---|---|---|---|---|---|---|
| **Comet assay** | *Area* | Wood | Urban | -0.066 ± 0.127 | -0.518 | 0.608 |
| | | Wood | Agri 1 | 0.103 ± 0.133 | 0.777 | 0.443 |
| | | Wood | Agri 2 | 0.070 ± 0.116 | 0.604 | 0.550 |
| | *Sampling year* | 2021 | 2022 | -1.176 ± 0.084 | -14.006 | <0.0001* |
| | *Sampling day* | | | 0.130 ± 0.051 | 2.535 | 0.015* |
| | *Body mass* | | | 0.001 ± 0.024 | 0.058 | 0.954 |
| **Total ENAs** | *Area* | Wood | Urban | 0.516 ± 0.083 | 6.199 | <0.0001* |
| | | Wood | Agri 1 | 0.544 ± 0.092 | 5.931 | <0.0001* |
| | | Wood | Agri 2 | 0.555 ± 0.078 | 7.089 | <0.0001* |
| | *Sampling year* | 2021 | 2022 | -0.184 ± 0.056 | -3.273 | 0.00107 * |
| | *Sampling day* | | | -0.150 ± 0.033 | -4.539 | <0.0001* |
| | *Body mass* | | | 0.045 ± 0.032 | 1.404 | 0.160 |
| **Bud nuclei** | *Area* | Wood | Urban | 0.606 ± 0.101 | 6.033 | <0.0001* |
| | | Wood | Agri 1 | 0.607 ± 0.111 | 5.453 | <0.0001* |
| | | Wood | Agri 2 | 0.615 ± 0.095 | 6.483 | <0.0001* |
| | *Sampling year* | 2021 | 2022 | -0.158 ± 0.068 | -2.315 | 0.0207 * |
| | *Sampling day* | | | -0.147 ± 0.040 | -3.675 | 0.00024* |
| | *Body mass* | | | 0.042 ± 0.037 | 1.12 | 0.263 |
| **Segmented nuclei** | *Area* | Wood | Urban | -0.254 ± 0.174 | -1.466 | 0.143 |
| | | Wood | Agri 1 | 0.162 ± 0.191 | 0.849 | 0.396 |
| | | Wood | Agri 2 | -0.004 ± 0.161 | -0.023 | 0.982 |
| | | Urban | Agri 1 | 0.416 ± 0.194 | 2.141 | 0.032* |
| | | Urban | Agri 2 | 0.251 ± 0.176 | 1.427 | 0.154 |
| | *Sampling year* | 2021 | 2022 | -0.646 ± 0.117 | -5.518 | <0.0001* |
| | *Sampling day* | | | -0.164 ± 0.067 | -2.458 | 0.014 * |
| | *Body mass* | | | 0.021 ± 0.067 | 0.312 | 0.755 |

| | | | | | | |
|---|---|---|---|---|---|---|
| **Notched nuclei** | *Area* | Wood | Urban | 0.979 ± 0.179 | 5.485 | <0.0001* |
| | | Wood | Agri 1 | 0.693 ± 0.203 | 3.419 | 0.00063* |
| | | Wood | Agri 2 | 0.660 ± 0.173 | 3.826 | 0.00013* |
| | *Sampling year* | 2021 | 2022 | -0.289 ± 0.121 | -2.4 | 0.0163* |
| | *Sampling day* | | | -0.112 ± 0.071 | -1.571 | 0.116 |
| | *Body mass* | | | 0.178 ± 0.072 | 2.479 | 0.013* |
| **Kidney nuclei** | *Area* | Wood | Urban | 0.186 ± 0.118 | 1.578 | 0.114 |
| | | Wood | Agri 1 | 0.387 ± 0.130 | 2.984 | 0.003* |
| | | Wood | Agri 2 | 0.475 ± 0.110 | 4.326 | <0.0001* |
| | | Urban | Agri 1 | 0.201 ± 0.131 | 1.535 | 0.125 |
| | | Urban | Agri 2 | 0.289 ± 0.116 | 2.488 | 0.013* |
| | *Sampling year* | 2021 | 2022 | 0.102 ± 0.080 | 1.28 | 0.200 |
| | *Sampling day* | | | -0.183 ± 0.047 | -3.882 | 0.0001* |
| | *Body mass* | | | 0.039 ± 0.047 | 0.838 | 0.402 |
| **MN** | *Area* | Wood | Urban | 0.843 ± 0.408 | 2.064 | 0.040* |
| | | Wood | Agri 1 | 1.292 ± 0.475 | 2.717 | 0.007* |
| | | Wood | Agri 2 | 0.675 ± 0.410 | 1.646 | 0.0990 |
| | *Sampling year* | 2021 | 2022 | -1.614 ± 0.304 | -5.313 | <0.0001* |
| | *Sampling day* | | | -0.307 ± 0.140 | -2.187 | 0.029* |
| | *Body mass* | | | -0.177 ± 0.138 | -1.283 | 0.200 |

**Table 4.14**. Outcomes of linear mixed models used to examine effects of each area on neurological functions in great tit (*Parus major*) nestlings sampled in 2021 and 2022. *p< 0.05, indicate significant difference.

| Variable | Factor | Reference Level | Level | Coefficient Estimate ± SE | *t* | *p* |
|---|---|---|---|---|---|---|
| **BChE activity** | *Area* | Wood | Urban | 0.121 ± 0.296 | 0.409 | 0.686 |
| | | Wood | Agri 1 | 0.024 ± 0.332 | 0.073 | 0.942 |
| | | Wood | Agri 2 | 0.091 ± 0.280 | 0.326 | 0.747 |
| | *Sampling year* | 2021 | 2022 | 0.621 ± 0.202 | 3.074 | 0.004* |
| | *Sampling day* | | | 0.015 ± 0.028 | 0.537 | 0.595 |
| | *Body mass* | | | 0.054 ± 0.060 | 0.903 | 0.370 |
| **CaE activity** | *Area* | Wood | Urban | 0.100 ± 0.232 | 0.43 | 0.671 |
| | | Wood | Agri 1 | -0.001 ± 0.254 | -0.004 | 0.997 |
| | | Wood | Agri 2 | -0.186 ± 0.214 | -0.87 | 0.392 |
| | *Sampling year* | 2021 | 2022 | 0.464 ± 0.158 | 2.936 | 0.007* |
| | *Sampling day* | | | 0.002 ± 0.020 | 0.109 | 0.914 |
| | *Body mass* | | | 0.060 ± 0.043 | 1.411 | 0.162 |

***Oxidative status alterations***

The highest levels of antioxidants were recorded in the agri 1 area with a statistical significant difference respect to urban area, which showed the lowest values. No statistical differences were observed between the two agricultural areas (Table 4.10, Figure 4.21).

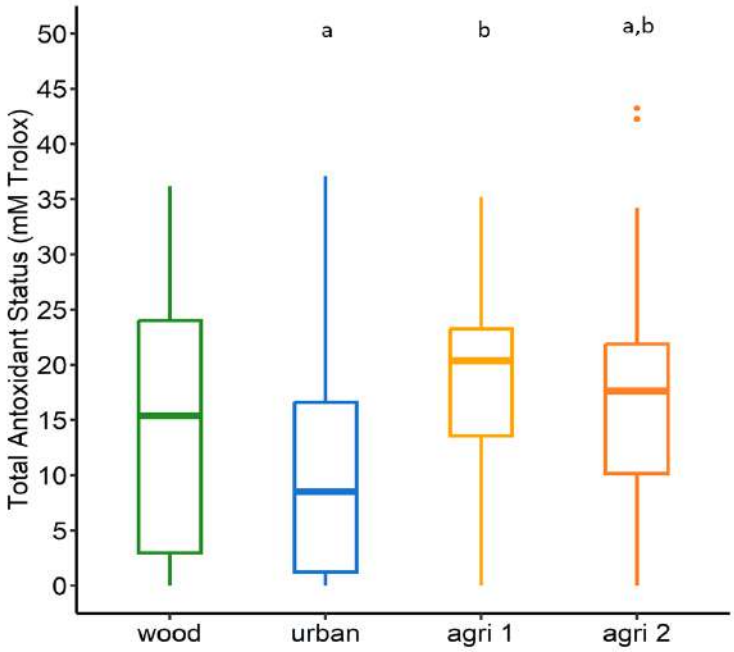


**Figure 4.21.** TAS measured in the blood of great tit (*Parus major*) nestlings sampled in different study areas during 2021 and 2022. Different letters indicate statistically significant differences among areas ($p<0.05$). Results are presented as median ± SE.

The presence of contaminants can induce an increase of pro-oxidants (Dimitrova-Shumkovskaand et al., 2022) and the organism can respond with an up-regulation of antioxidants (Beaulieu and Costantini, 2014). The highest concentration of antioxidants measured in nestlings from agri 1 could suggests that these birds are subjected to some environmental pressure. It should be pointed out that the agri 1 area, Oasi Ca' di Mezzo, is an artificial phytodepuration site surrounded by intensive cultivation of wheat and corn. This site collects the wastewater of Altipiano canal that drains water from upstream farmland and was created to remove nitrogen and phosphorus before waters are released into Venice lagoon. Therefore, we can suppose the presence of plant protection products in agri 1 area. Moreover, this area is also bordered by the Bacchiglione river and it is nearby the Brenta river, known to be contaminated by PFAS (Sciancalepore et al., 2021; Valsecchi et al., 2015) and by other industrial compounds. PFOS levels measured in unhatched eggs collected in Agri 1 area showed the highest values, if compared to the other study sites, also ∑OCPs and ∑HCHs levels in

eggs were among the highest found in our study. Buytaert et al. (2023) studied the relation between plasma PFAS concentration and the oxidative status in adult great tits living in two sites near a fluorochemical manufacturing facility (Belgium), finding that the total antioxidant capacity was higher near the plant site, where also PFAS showed highest values.

Regarding our study, the lowest levels of TAS were recorded in the urban area. Salmón et al. (2018) conducted a research on four common passerine bird species (*Cyanistes caeruleus, Parus major, Passer domesticus, Passer montanus*) living along a gradient of NOx and urbanisation levels in southern Sweden. Individuals living in areas with higher urbanization level had also higher levels of total antioxidant capacity respect to those from lower degree of urbanization. In addition, Isaksson and collaborators (2007) found similar results. They measured the plasma antioxidant activity (TAA) in adult and nestling *Parus major* sampled in urban and rural sites. Adult urban birds had higher antioxidant activity than adult from rural birds and this increase could be a consequence of urban environmental stress. On the contrary, Herrera-Dueñas et al. (2014; 2017) showed a reduction of the total antioxidant capacity in house sparrow urban individuals compared to those from a rural area. These results could be due to an increase in oxidation degree and simultaneously lack of antioxidant defence response of individuals of urban area. Namely, the total antioxidant capacity has reached its maximum capacity and is decreased by the pro-oxidative effects of pollution. Our urban great tits have shown low antioxidant capacity, indicating a possible physiological stress condition. Indeed, the city of Padua was identified as city with high pollution rate (Ottaviano et al., 2022). The animals probably are not able to cope with a pro-oxidative condition induced by environment or by inflammatory processes, which are combined with increasing heterophiles in blood. This is consistent with results of Costantini and Dell'Omo (2006), who showed that immune challenge leads to a decrease of total antioxidant defences.

***Porphyrins levels***

Coproporphyrins showed the lowest levels in agri 1 and differed significantly from wood area; similarly, protoporphyrins level of agri 2 was significantly lower than those measured in great tits from wood. In addition, protoporphyrins resulted the most abundant porphyrin class compared to copro- and uroporphyrins. Total porphyrin levels were lower in both agricultural areas with a statistically significant difference compared to wood (Table 4.11, Figure 4.22).

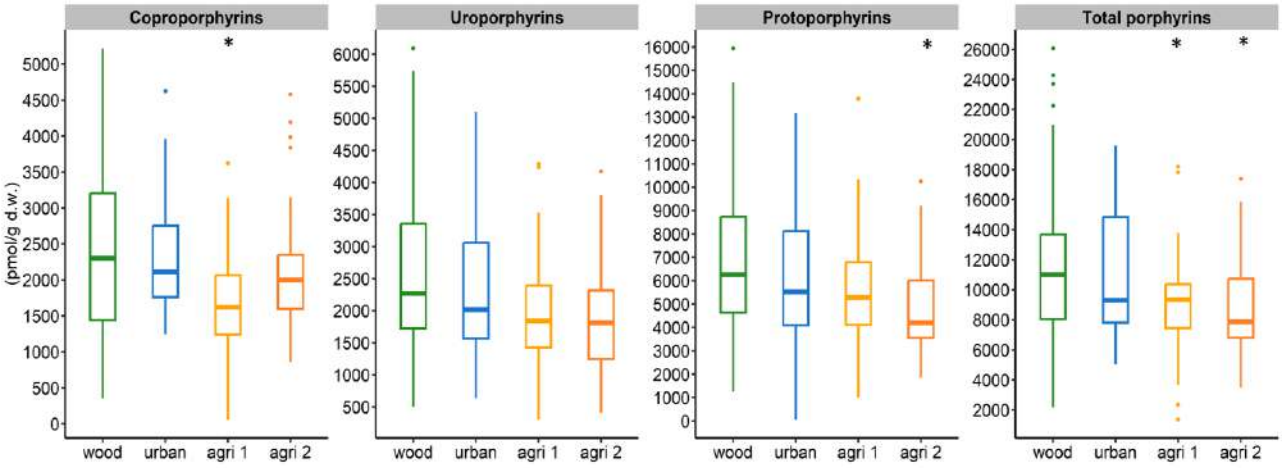


**Figure 4.22.** Levels of the different porphyrins (copro-, uro- and protoporphyrins) and total porphyrins measured in excreta of great tit (*Parus major*) nestlings sampled in different study areas during 2021 and 2022 * indicates a statistically significant difference respect to wood area ($p<0.05$). Results are presented as median ± SE.

The fundamental physiological role of porphyrins is linked to the synthesis process of heme of which they are intermediate metabolites or oxidation byproducts of intermediate metabolites. Their accumulation may suggest the exposure to heavy metals or to other toxic compounds such as dioxins, PCBs etc. To our knowledge, no published studies are available up to now on porphyrin levels in excreta of *Parus major.* Levels of total porphyrins measured in *Parus major* from wood and urban areas are almost the double than those measured in industrial (8600 pmol/g d.w.) and agricultural (6600 pmol/g d.w.) areas investigated by Casini et al. (2001), that analysed excreta samples of three seabird species (*Pelecanus thagus, Phalacorax olivaceus, Larus dominicanus*) from three Chilean areas with different human impact (control, heavy industry and intensive agriculture sites). Moreover, the total porphyrins levels detected in great tits from wood area are higher than those found in laboratory studies on Japanese quail (*Coturnix coturnix japonica*) exposed to PCB and methylmercury (Fossi et al., 1996; Leonzio et al., 1996). The results of our research suggest that great tits from all sampling sites are probably exposed to contaminants that interfere with the enzymatic processes responsible for haem biosynthesis, leading to accumulation of porphyrins. In line with Casini et al (2001) results, we also found the predominance of protoporphyrins respect to the other two classes of porphyrins analysed. This trend is respected for all the study areas. Our values of protoporphyrins were about three times higher than those recorded in Humboldt penguins living nearby an industrialized area (Celis et al., 2014). An accumulation of protoporphyrins could be due to Pb intoxication, as reported by Casini et al. (2003). This metal can interfere with the iron transfer mechanisms causing therefore a reduction of availability of Fe2+; so, the Zn can substitute iron as a substrate and forms zinc-protoporphyrin instead of heme. We recorded the highest levels

of Pb in feathers and highest total porphyrin values in excreta in great tits coming from Euganean Hills. Although this is a wooded area that may be considered less impacted by anthropic activities, the great tit nestlings from Euganean Hills could be exposed to pollution from Padova city, around 15 km away. Indeed, this city has been recognized as one of the most polluted cities in Europe by the European Environmental Agency (Thunis et al., 2017). It is interesting to note that Scartezzini and collaborators (2021) found that in Euganean Hills, the mean concentration of PM2.5 was similar to those of Padova center. Sicolo et al. (2009) found a close connection with increasing concentration of protoporphyrins and PM2.5 in individuals of *Columba livia* sampled in Milan city during winter. The supposed presence of contaminants in the wood area can be linked to the fact that the trees canopies play an important role in the interception of atmospheric heavy metals and polycyclic aromatic hydrocarbons depositions (De Nicola et al., 2015). Finally, it should also be underlined that Euganean Hills are not far away from sites with known PFAS contamination (Mastrantonio et al., 2018). Indeed, the analysis of great tit eggs that we sampled in Euganean Hills showed the higher level of longPFCA compared to the other study areas. It is also worth to mention that porphyrin production can also be due to a general mechanism of oxidative stress, able to oxidate porphyrin precursors.

***Immunotoxicity***

*Complement system activity*

The complement system activity measured in *Parus major* individuals from agri 2 was significantly higher than those recorded in nestlings from wood and agri 1 areas (Table 4.12, Figure 4.23).

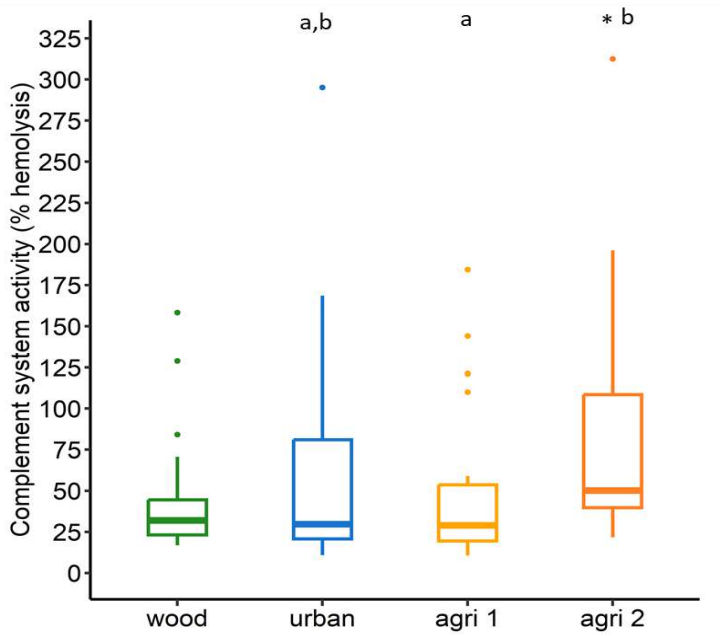


**Figure 4.23.** Complement system activity measured in blood of great tits (*Parus major*) nestlings sampled in different study areas during 2021 and 2022. Different letters indicate statistically significant differences among areas. * indicates a statistically significant difference respect to wood area ($p<0.05$); different letters indicate statistically significant differences among areas ($p<0.05$). Results are presented as median ± SE.

The immune system allows animals to counter against invading pathogens and established infections. It comprises innate immunity, functioning as a non-specific first line of defense, and adaptive immunity, which offers a specific protection and immunological memory (Vermeulen et al., 2017). Specifically, the innate immunity includes disease-resistance mechanisms that are present in the organism independently of a prior antigenic stimulation. These mechanisms are actuated by diverse components of the innate immunity that include complement proteins (complement system), acute-phase proteins, phagocytic cells, such as macrophages, monocytes and granulocytes. Furthermore, also natural killer cells are part of the innate immunity which are involved in surveillance processes against cancers and tumours (Fairbrother et al., 2004; Vallverdú-Coll et al., 2019). Environmental contaminants can impair immune system functions (Marteinson et al., 2017), thus evaluating of proper functioning of immune components can provide information on the contaminants effects on animals. The function of complement system is to lyse foreign cells. A good wild bird survival is ensured by a robust immune response that permit a high levels of hemolysis with increased complement levels (Vennum et al., 2019; Hegemann et al., 2017). The high values of the complement system emerged in the individuals of agri 2 area could suggest that the innate immune response of these birds is stronger than that of great tits from the other study areas. We could hypothesize a reduced immune capacity in the individuals from wood and agri 1 areas.

Vermeulen et al. (2015) found a negative correlation between haemolytic response and lead concentration in great tit nestlings. A reduction of complement system activity was also observed in common kestrel nestlings from urbanised areas (Wemer et al., 2021). We can advance the hypothesis that individuals from wood and agri 1 areas had an impairment of the complement system activity, this impairment can be due to multiple stressors including environmental contamination. Specifically, wood area could be affected by urban and industrial pollutants that can reach this area by natural agents such as rain or wind. Our data on longPFCA levels in unhatched eggs confirm that this area is affected by this kind of contamination; the precursors of these compounds are transported from their emission areas and can settle in tree-rich areas, as the leaves capture such extremely volatile compounds. With the deposition of plant material in the soil, longPFCA precursor become available and can be assumed by feeding from the great tit, as it eats soil organisms, such as earthworms. The agri 1 area, as already mentioned, could be subjected to the presence of agrochemical, applied in the surrounding crops, and industrial compounds that can be present and transported in the nearby Brenta and Bacchiglione rivers. Also, PFOS levels measured in eggs of the animals showed the highest values among all the areas investigated.

*Respiratory burst*

Regarding the respiratory burst, no statistically significant differences emerged among areas (Table 4.12, Figure 4.24).

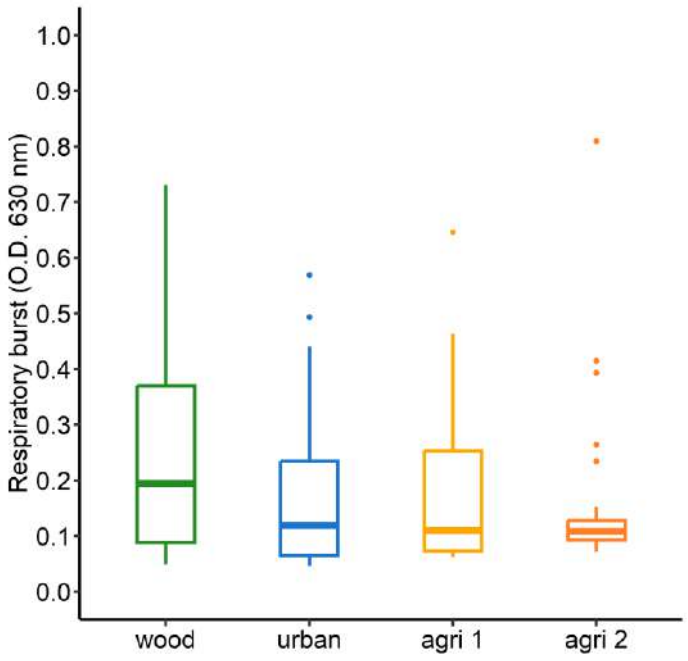


**Figure 4.24.** Respiratory burst process measured in blood of great tits (*Parus major*) nestlings sampled in different study areas during 2021 and 2022. Results are presented as median ± SE.

The respiratory burst process represents the activation of innate immune response and involves the rapid release of reactive oxygen species by phagocytes. The ROS destroy microorganisms by damaging their macromolecules but are not pathogen-specific and thus can also damage host tissues, leading to immunopathology (Meitern, 2016). If not efficiently counteracted by the antioxidant defenses of the organism, ROS can accumulate and cause oxidative stress (Costantini, 2008). The respiratory burst test has been applied for the first time on adult free-living great tits to investigate the responses of cellular oxidative burst in animals with different age; a pick of cellular respiratory burst in midlife individuals and a decrease in older birds was observed (Těšický et al., 2021). Rainio et al. (2015) showed no relation between phagocytosis activity and lead concentrations in nestling great tits population living near a metal smelter. However, the data recorded in the nestlings of our areas are not comparable with those of the above studies since different methodologies were applied.

*White blood cells count*

Results of differential white blood cells (WBCs) count are shown in Table 4.12 and Figure 4.25. The number of heterophiles were significantly higher in *Parus major* individuals from urban and agri 2 areas compared to wood site. Eosinophils were significantly more numerous in urban area than agri 1. Lymphocytes were significantly less abundant in urban nestlings than those from wood and agri 1 areas. Monocytes did not differ among study areas. Basophil cells were not found in any analysed sample.

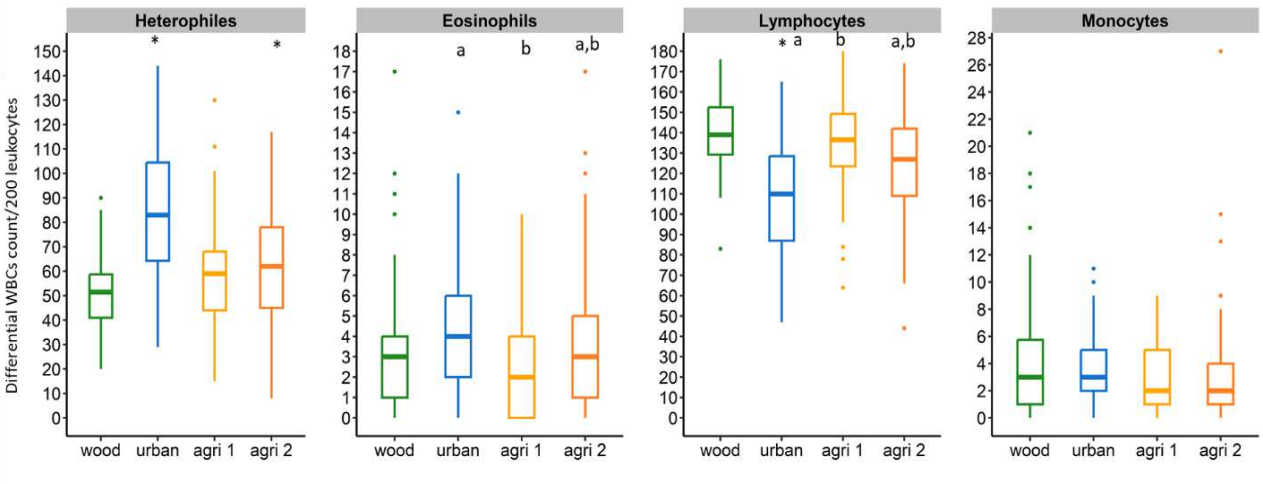


**Figure 4.25.** Differential WBCs count evaluated in blood of great tits (*Parus major*) nestlings sampled in different study areas during 2021 and 2022. * indicates a statistically significant difference with wood ($p<0.05$); different letters indicate statistically significant differences among areas. Results are presented as median ± SE.

H/L ratio resulted significantly higher in urban nestlings than those from wood and agri 1 areas (Table 4.12, Figure 4.26).

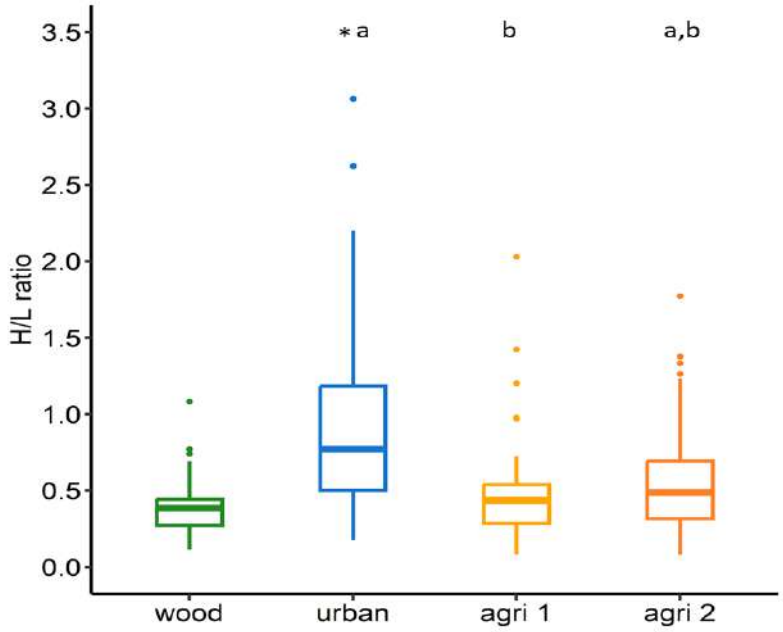


**Figure 4.26.** H/L ratio measured in blood of great tits (*Parus major*) nestlings sampled in different study areas during 2021 and 2022. * indicates a statistically significant difference with wood ($p<0.05$); different letters indicate statistically significant differences among areas. Results are presented as median ± SE.

White blood cells, involved in the immune response, are distinguished into granulocytes, characterized by the presence of specific granules and agranulocytes, cells without granules. The granulocytes include heterophils, eosinophils and basophils, while the second ones comprehend monocytes, which mature in macrophages, and lymphocytes that represent a form of cellular-mediated immune defense of the adaptive system (Vallverdú-Coll et al., 2019). Heterophils are phagocytic cells that proliferate in circulation in response to infections, inflammation as well as stress condition (Skwarska, 2018). Eosinophil has a role in inflammation process and in defense against pathogens. The function of basophils is still not clear; however, these cells probably mediate the early inflammatory response (Davis et al., 2008; Maxwell et a., 1993). Monocytes are the other phagocytic cells involved in the defense against infections and bacteria (Campbell, 1995). Lymphocytes play a role in cell-mediated adaptive immunity (T-cells) and humoral adaptive immunity (B-cells). They are involved in production of immunoglobulins and modulation of immune defense (Minias 2019; Campbell 1996). The differential WBCs count allow to gets indications on potential infection and inflammation conditions (Campbell 1995) of the animal and can indicate possible alteration of the immune functions; moreover, the evaluation of leucocyte profile,

especially H/L ratio, can also provide information on exposure to contaminants and habitat quality (Cid et al., 2018; Skwarska, 2018).

H/L ratio is often used as stress indicator, which increase as a response to stressors (Latimer et al., 2020). When an animal is exposed to environmental stressors, an increase of glucocorticoid hormone levels occurs. This leads to changes in the leukocyte profile, wherein circulating lymphocytes adhere to blood vessels walls and subsequently migrate from the bloodstream into other tissues, such as lymph nodes, spleen and bone marrow. This migratory process results in a substantial reduction in their circulating abundance. In contrast, glucocorticoids also prompt an increase in the influx of heterophiles from the bone marrow into the bloodstream and dampen the movement of heterophiles from the bloodstream to other compartments (Davis et al., 2008).

The mean values of heterophils recorded in great tits of the present study are higher than values included in an online database for passeriforms, while lymphocytes data were in line (wildlifehematology.uga.edu).

The highest increase of heterophiles and a simultaneous decrease of leukocytes recorded in the urban *Parus major* nestlings are almost four times higher than those of Eurasian tree sparrow (*Passer montanus*) from a Chinese area with higher levels of $PM_{2.5}$ and $PM_{10}$ (Li et al., 2021). Our data are consistent with results of a study conducted on sub-urban wild great tit (*Parus major*) population monitored for twelve years. The authors showed an increase of H/L ratio with increasing levels of Pb in the blood (Bauerová et al., 2020). Bauerová et al. (2017) reported a condition of long-term stress in great tit population from urban centers where higher values of H/L ratio were recorded with increasing feather heavy metal contamination. Besides the great tit, other passeriform species sampled in peri-urban and urban areas characterized by anthropogenic noise and high carbon monoxide (CO) emission showed an increase of H/L ratio (Ribeiro et al., 2022). We can hypothesize that the great tit living in the urban area are subject long-term stress or inflammation conditions (Campbell and Ellis, 2007). Urban great tits showed also highest values of eosinophils. In general, eosinophilia is linked with a generalized inflammation condition (Mitchell and Johns, 2008). Perhaps this inflammation condition could be due to the presence of PMs and other toxic contaminants such as PAHs. In fact heavy metals (Cd, Pb and Hg), OCPs and HCHs, showed higher values in urban area respect to the agricultural areas.

***Genotoxicity***

*Comet assay*

Great tit nestlings from the different study areas showed no significant difference of comet assay values (Table 4.13, Figure 4.27).

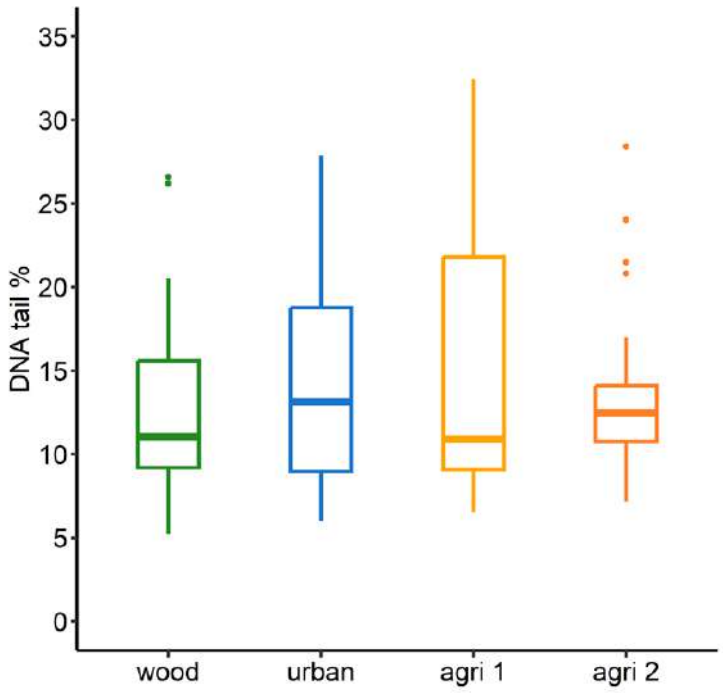


**Figure 4.27.** DNA tail % measured in blood of great tits (*Parus major*) nestlings sampled in different study areas during 2021 and 2022. Results are presented as median ± SD.

The comet assay allows to measure DNA strand breaks, which can be due to breakage and repair processes in eukaryotic cells (OECD, 2016). To date, this test was never applied on *P. major* and no basal levels are available for the species object of our study.

Montoya et al. (2020) investigated the presence of DNA damage in free-living spotless starlings living in a site located near to a Spanish protected area, founding a DNA damage higher (27% of tail DNA) than those measured in our area study. Similarly, a multi-specie birds' study (Galván et al., 2014), which individuals were sampled in different sites surrounding the Chernobyl nuclear power plant, showed 35.37% of DNA tail. Specimens, such as *Columbia livia* and *Hirundo rustica,* collected in different contaminated areas showed higher values of comet assay (Sicolo et al. 2010; Bonisoli-Alquati et al., 2010).

However, in order to make a more complete assessment of possible genotoxic effects on the different populations of great tits, further studies are needed to carry out baseline reference values. In general, we could consider an average DNA damage value of 12%, measured in the wood area, as

homeostatic values of the species; this agrees with the international guidelines on comet assay described in International Workshop on Genotoxicity Testing (Kirkland et al., 2007).

*ENA assay*

As shown in Table 4.13 and Figure 4.28, the total ENAs values recorded in nestlings from urban, agri 1 and agri 2 were statistically different than those of wood area. The number of bud and notched nuclei counted in nestlings from urban, agri 1 and agri 2 areas was significantly higher than those of wood (Figure 4.29). Segmented nuclei were significantly more abundant in agri 1 than urban. Kidney nuclei values were significantly higher in pullets from agri 1 and agri 2 than those from wood. For this anomaly, a significant difference emerged also between agri 2 and urban areas. Finally, the number of micronuclei was significantly higher in great tits from urban and agri 1 areas than those from wood. It should be noted that bud nuclei are the predominant anomaly among those counted.

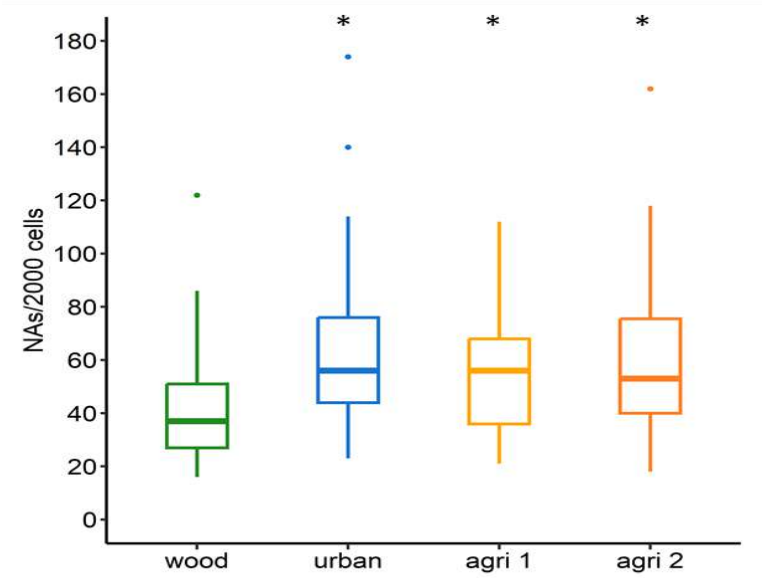


**Figure 4.28.** Total erythrocytes nuclear abnormalities evaluated in blood of great tits (*Parus major*) nestlings sampled in different study areas during 2021 and 2022. * indicates a statistically significant difference with wood ($p<0.05$). Results are presented as median ± SE.

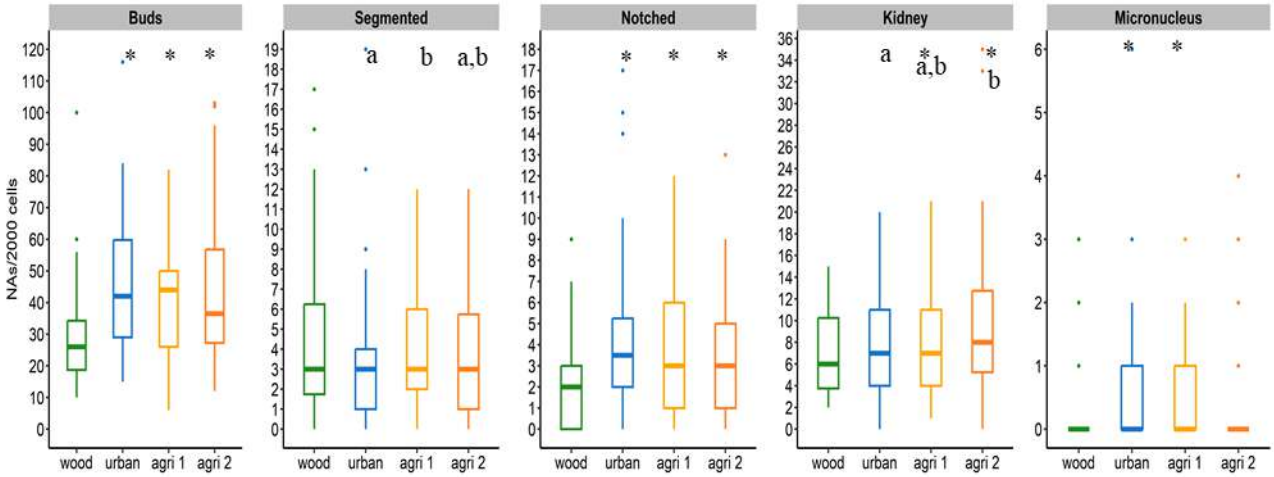


**Figure 4.29.** Erythrocyte nuclear abnormalities divided by nucleus shape, evaluated in great tits (*Parus major*) nestlings sampled in different study areas during 2021 and 2022. * indicates a statistically significant difference with wood ($p<0.05$); different letters indicate statistically significant differences among areas. Results are presented as median ± SD.

Environmental contaminants (such as heavy metals, pesticides, PAHs and POPs in general) can affect DNA integrity causing a genomic damage in the erythrocyte nucleus (Louzon et al., 2019) and this damage can be exhibited as erythrocyte nuclear abnormalities. The nuclear abnormalities are chromosomal damage due to loss or alteration of mitotic spindle induced by pollutants; some abnormalities can also be caused by increasing permeability of the nuclear membrane of the contaminants (Viana et al., 2018; Hatch et al., 2013). The nucleous abnormalities might probably respond more rapidly to pollution than micronuclei formation and could then be used as sensitive biomonitors also in avian species. Erythrocyte abnormalities could also show a varied response to the diversity of pollutants. Therefore, the total frequency of erythrocyte abnormalities and the number of abnormality types per individual can be used for analysis, as these variables would better reflect the effects of diverse pollutants.

A study conducted by Drahulian et al. (2018) on *Parus major* nestlings from a Ukrainian forest showed a micronuclei frequency (3.4 ± 0.45 ‰) higher than this of great tits from our study areas. Quero and collaborators (2016) found MN frequency in different passeriformes from a biosphere reserve in Argentina that were half of what we found in urban and agri 1 areas. Comparing to literature data, the values of MN found in the urban and agri 1 areas permit to suppose the presence of genotoxic compounds in these areas.

Regarding the bud anomalies, different authors observed lower values, measured in contaminated areas, than those counted in our study (Kursa et al., 2005; Frixione et al., 2022). The total anomalies frequency measured in our urban and agricultural areas are higher in comparison to the frequency

observed by Kursa et al (2005) and Tomazelli et al. (2022) in industrial and urban areas.

Feathers collected from urban specimens showed the highest levels of heavy metals, in particular Hg, Cu and Pb. It's known that heavy metals have genotoxic effects on organisms (Turna Demir and Yavuz, 2020; Kopp et al., 2018) and that can induce DNA damage secondary to oxidative stress (Espín et al., 2014). Is worth also to underline that the highest levels of PFAS and OCs were found in abandoned eggs from urban and agri 1 areas. Presence of pesticides in agri 1 site can in fact be another possible cause of genotoxic effects in birds (Gill et al., 2018; Cocco, 2022).

Low DNA damage (Comet assay) values and simultaneous high levels of chromosomic aberrations (ENA assay) could be due to the fact that the responsible compounds for the genotoxic effects are substances able to avoid the biotransformation processes and cause irreversible genotoxic effects. It could also be hypothesized that in this specific case, the comet assay was not sufficiently sensitive and specific to reveal differences between individuals from the different study areas. In contrast, the high ENA assay values were found to be in line with the responses of other biomarkers and the presence of contaminants, revealing that urban and agri 1 areas were the most impacted sites.

***Neurotoxicity***

Neurotoxic effects evaluated through BChE and CaE activities did not show significant differences among study areas (Table 4.14, Figure 4.30).

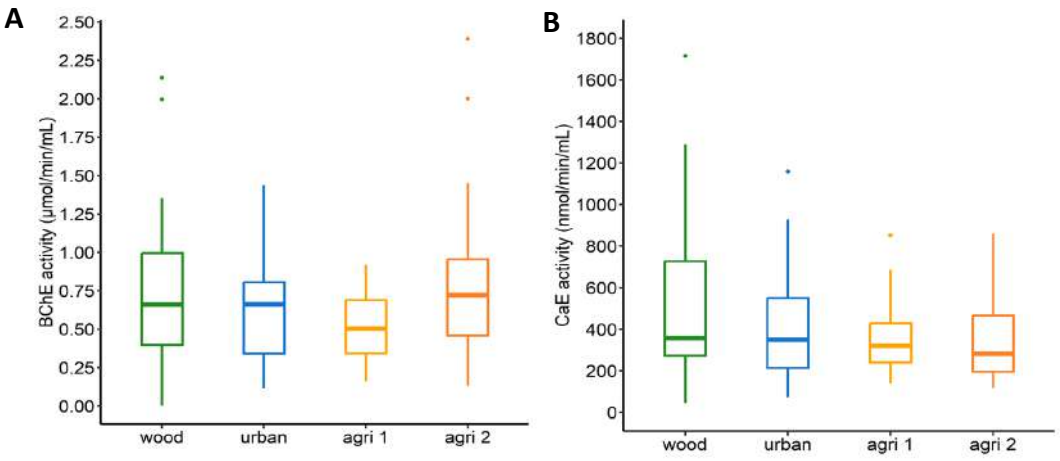


**Figure 4.30.** BChE (A) and CaE (B) activities measured in blood of great tits (*Parus major*) nestlings sampled in different study areas during 2021 and 2022. Results are presented as median ± SE.

Values of BChE recorded in *P.major* individuals from our study areas are double or even almost ten times higher than those shown by various works of Norte et al. (2008a, 2008b, 2010) conducted on

adult and nestling great tits living in woodlands, suburban or industrial areas. Our BChE data were also twice than those showed by Tsarpali and collaborators (2020) in specimens of free-living Eleonora´ s falcons. Other monitoring studies report lower BChE values in avian species than those recorded in our great tits (Santos et al., 2012; Strum et al., 2008).

The CaEs are ubiquitous enzymes with the main function of hydrolyzing various endogenous and exogenous esters compounds, included environmental contaminants. Thus, they are both involved in the phase I metabolism processes (converting substances into more polar chemicals, increasing their solubility and facilitating their excretion) and protecting AChE by providing alternative sites for insecticides binding (Casey et al., 2013; Arufe et al., 2010). However, the activity of these enzymes can be inhibited by pesticides and other contaminants (Narváez et al., 2016; Bjedov et al., 2023). A 30 % inhibition of CaE activity has measured in *P. major* individuals (Cordi et al., 1997) exposed to dimethoate spray drift.

We can suppose that the great tit nestlings from our study areas are not exposed to compounds which impair the BChE and CaE activities.

*Effects of sampling year on biomarkers*

Regarding immunotoxicity, oxidative stress and neurotoxicity, the biomarker values were significantly higher in 2022 than in 2021 (Tables 4.10, 4.12, and 4.14). No significant difference was found between years for porphyrins, except for coproporphyrins whose levels were higher in 2021 than in 2022 (Table 4.11). Eosinophil cells tended to be higher in 2022 than 2021, while the number of monocytes were significantly lower in individuals of 2022 (Table 4.12). Nestlings of 2021 showed significantly higher DNA fragmentation and nuclear abnormalities, evaluated with comet and ENAs assays respectively, than those of 2022 (Table 4.13).

*Effects of body mass on biomarkers*

The TAS was negatively correlated with body mass. Therefore, heavier pullets showed lower antioxidant values (Table 4.10). Heavier nestlings showed higher complement system activity (Table 3). Heavier nestlings presented lower numbers of heterophiles and higher lymphocytes and monocytes (Table 4.12b). In addition, H/L ratio was negatively correlated with body mass (Table 4.12). All these data permit to suppose that heavier animals have a greater ability to react to the presence of contaminants, implementing its own mechanisms of oxidative and immune defence. It could be hypothesized that great tits with a greater body mass were in better physical condition (Poliserpi et al., 2023; Eng et al., 2019; Bernat-Ponce et al., 2023), therefore not particularly affected

by stress conditions that required a high antioxidant response. Surprisingly, we found a positive correlation between notched nuclei and body mass. This may suggest that specific contaminants caused this genotoxic effect even in individuals with good physical and physiological conditions, as shown above by most of the applied biomarkers. A similar result was also found by Tomazelli et al. (2022) where omnivorous birds with better body condition had increased nuclear abnormalities numbers. No effects of the body mass were observed on neurotoxicity responses.

*Effects of sampling day on biomarkers*

No effects of the sampling day was found on all the biomarkers, except for genotoxicity biomarkers (Tables 4.10, 4.11, 4.12 and 4.14). In particular, comet assay was positively correlated with sampling day, which means that pullets sampled forward during the breeding season had higher DNA fragmentation values. It emerged also that pullets sampled at the beginning of the breeding season had higher total ENAs values (Table 4.13). It could be hypothesized that the chromosomal aberrations found in 15-day-old pullets have been inherited by the mother, and that only at a later time they implement reparative mechanisms.

#### 4.4.2.2. Behavioural traits

Behavioural traits investigation was conducted for three years (2021-2023) on 295 great tit nestlings inhabiting the study areas (wood, urban, agri 1 and agri 2). We studied the nestlings' behaviour by measuring their breath rate and agitation state and evaluating the tonic immobility, before performing the blood sampling. Outcomes of linear mixed models are reported below (Table 4.15).

**Table 4.15.** Outcomes of linear mixed models used to examine effects of each area on behaviour of great tit (Paru*s major*) nestlings sampled in 2021, 2022 and 2023. * indicates a significant difference (p< 0.05).

| Variable | Factor | Reference Level | Level | Coefficient Estimate ± SE | *t/z* | *P* |
|---|---|---|---|---|---|---|
| **Breath rate** | *Area* | Wood | Urban | -1.521±2.737 | -0.556 | 0.581 |
| | | Wood | Agri 1 | -8.542±3.958 | -2.793 | 0.007* |
| | | Wood | Agri 2 | -7.269±2.889 | -2.516 | 0.014* |
| | | Urban | Agri 1 | -7.021±3.164 | -2.219 | 0.030* |
| | | Urban | Agri 2 | -5.749±2.976 | -1.932 | 0.057 |
| | *Sampling year* | 2021 | 2022 | 10.643±2.440 | 4.362 | 0.005* |
| | | 2021 | 2023 | 14.727±2.451 | 6.008 | 0.001* |
| | *Sampling day* | | | 2.616±1.216 | 2.151 | 0.035* |
| **Agitation state** | *Area* | Wood | Urban | 0.500±0.301 | 1.660 | 0.096 |
| | | Wood | Agri 1 | -2.279±0.342 | -0.815 | 0.415 |
| | | Wood | Agri 2 | 0.316±0.313 | 1.010 | 0.312 |
| | | Urban | Agri 1 | -0.779±0.349 | -2.231 | 0.026* |
| | | Urban | Agri 2 | -0.185±0.318 | -0.583 | 0.560 |
| | | Agri 1 | Agri 2 | 0.594±0.301 | 1.977 | 0.048* |
| | *Sampling year* | 2021 | 2022 | 0.377±0.269 | 1.402 | 0.161 |
| | | 2021 | 2023 | 0.957±0.267 | 3.581 | 0.003* |
| | *Sampling day* | | | -0.018±0.130 | -0.139 | 0.890 |
| | *Body mass* | | | 0.082±0.022 | 3.813 | 0.001* |
| **Tonic immobility** | *Area* | Wood | Urban | 0.148±0.278 | 0.531 | 0.596 |
| | | Wood | Agri 1 | 0.098±0.313 | 0.316 | 0.752 |
| | | Wood | Agri 2 | 0.045±0.288 | 0.155 | 0.877 |
| | *Sampling year* | 2021 | 2022 | 0.563±0.246 | 2.286 | 0.022* |
| | | 2021 | 2023 | 0.603±0.247 | 2.439 | 0.015* |
| | *Sampling day* | | | 0.133±0.119 | 1.118 | 0.264 |
| | *Body mass* | | | -0.066±0.018 | -3.663 | 0.002* |

*Breath rate*

Analysis of the breath rate data showed statistically significant differences among great tit populations living in the different study sites. Individuals from agri 1 and agri 2 areas resulted significantly less agitated than those from wood ($p<0.05$), and great tits from agri 1 also showed a significantly lower breath rate than nestlings from urban area ($p<0.05$) (Figure 4.31). We found an effect of the sampling year and sampling day (nestlings' age) on great tit breath rate (Table 4.15). Nestlings sampled in 2022 and 2023 showed higher breath rate than in 2021 ones ($p<0.05$) and individuals born later during the season were more agitated or frightened. The breath rate is

considered a physiological trait related to stress response and the differences that we found for breath rate in the different environments are consistent with other studies conducted on great tit (Torné-Noguera et al., 2014; Charmantier et al., 2017) individuals from urban and rural areas.

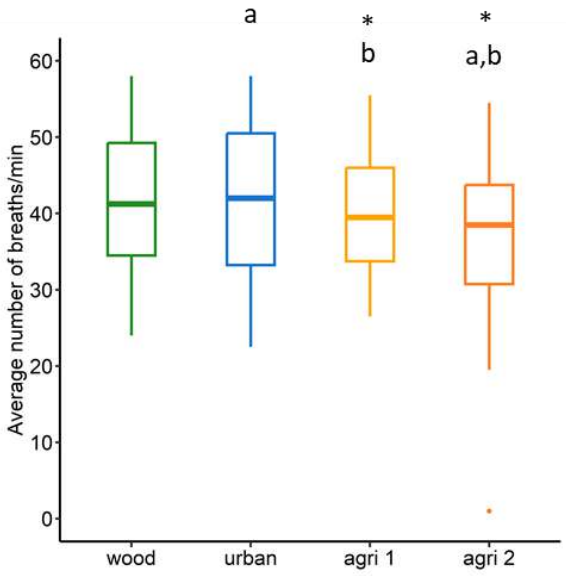


**Figure 4.31.** Breath rate measured in great tits (*Parus major*) nestlings during 2021, 2022 and 2023. * indicates a statistically significant difference respect to wood area ($p<0.05$); different letters indicate a statistically significant difference among areas ($p<0.05$). Results are presented as median ± SE.

*Agitation state*

The agitation state is considered as a measure of nestlings' agitation (Corti et al., 2017), thus similar to breath rate measurement. We found an effect of the different areas on the agitation state of great tit nestlings. Individuals from agri 1 were significantly less agitated than those from urban and agri 2 areas ($p<0.05$, Table 4.15, Figure 4.32). Furthermore, nestlings with higher body mass showed higher values of agitation state (Table 4.15).

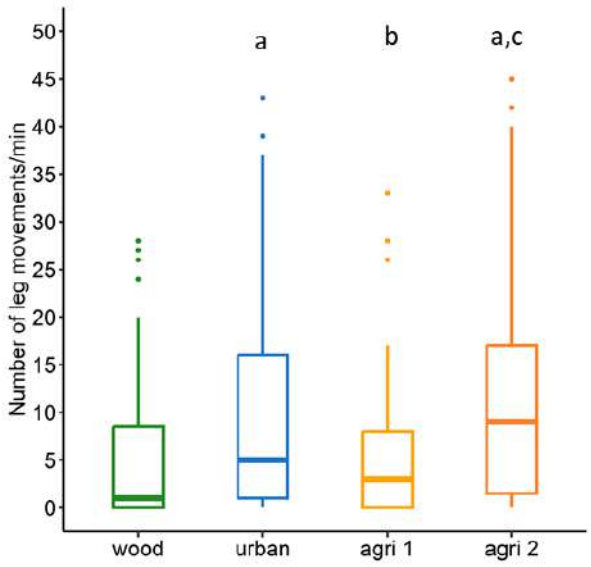


**Figure 4.32.** Agitation state measured in great tits (*Parus major*) nestlings during 2021, 2022 and 2023. Different letters indicate a statistically significant difference among areas ($p<0.05$). Results are presented as median ± SE.

*Tonic immobility*

The data analysis of tonic immobility showed no significant difference between great populations from the different study areas. On the contrary, nestlings sampled in 2022 and 2023 showed a significantly prolonged catatonic state than in 2021 ($p<0.05$, Table 4.15, Figure 4.33). An effect of body mass on tonic immobility was found. Individuals with a mean higher body mass had a less prolonged catatonic state ($p<0.05$ Table 4.15). Since the physiological conditions of an organism influence its behavior, it can be hypothesized that individuals heavier, and therefore in better health condition, assume more proactive and neophilic tendencies, namely a shorter duration of the catatonic state (Senar et al., 2017).

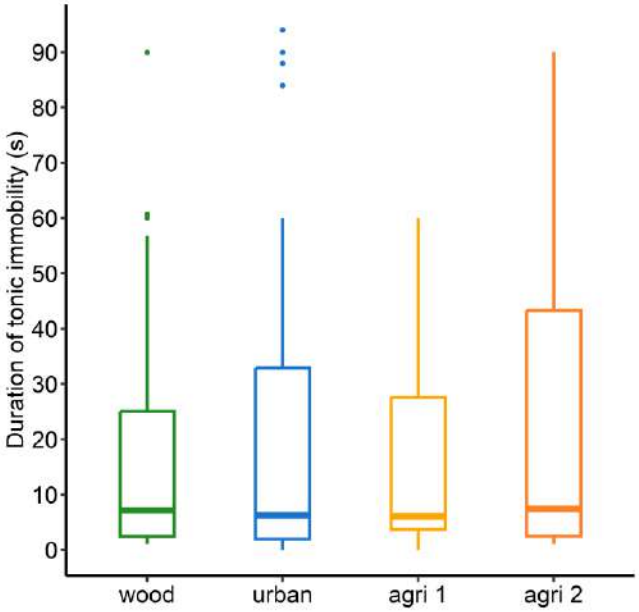


**Figure 4.33.** Tonic immobility measured in great tits (*Parus major*) nestlings during 2021, 2022 and 2023. Results are presented as median ± SE.

Considering all the conducted behaviour tests, we found that environments act on some aspects of the behaviour of great tits populations studied.

We found interesting correlations among behavioural traits with genotoxicity and neurotoxicity biomarkers and H/L ratio, and this can be related to the potential effects of multiple stressors characterizing the anthropized environments. Specifically, breath rate was positively correlated with the total ENAs (Estimate=2.584±1.210; t= 2.135; p=0.03) and BChE activity (Estimate=1.943±0.691; t= 2.812; p=0.007). Thus, nestlings more agitated showed a higher frequency of nuclear abnormalities and an elevated activity of the plasma enzyme BChE. The agitation state was negatively correlated with comet assay (Estimate=-0.490±0.235; z=-2.084; p=0.04) and positively with the H/L ratio (Estimate=0.329±0.163; z=2.012; p=0.04). High DNA fragmentation values indicate DNA damage, and the agitation state is a proxy of bold personality and is considered a stress response. Individuals with more timid and submissive personalities showed higher DNA damage, while those more agitate and with a bold personality showed higher H/L ratio that is a recognized index of physiological stress.

Regarding the tonic immobility we found a positive correlation with comet assay (Estimate=0.106±0.345; z=3.066; p=0.002) and a negative correlation with total ENAs (Estimate=-0.107±0.026; z=-4.118; p=0.004). Tonic Immobility is an expression of the emotional reactivity that indicates how an animal is able to react to environmental stimulus. It is the opposite of the agitation

score thus higher tonic immobility rate indicates a shyer personality. The positive correlation that emerged between tonic immobility and comet assay may suggest that individuals with more shy personalities had higher DNA strand breaks or repair sites. The negative correlation found between tonic immobility and ENAs assay indicates that individuals with a bold personality and proactive behaviour (low tonic immobility score) had higher chromosomal damage that may be induced by different contaminant classes compared to the ones that caused DNA strand breaks or repair sites evaluated through the comet assay.

#### 4.4.2.3. Reproductive performances

We investigated the reproductive performances for three years (2021 - 2023) by monitoring a total of 160 nest boxes distributed along the study areas. We evaluated different reproductive success parameters: laying date, clutch size, hatching and fledging successes. Below, mean, standard deviation, min and max values are summarised in the Table 4.16; outcomes of linear mixed models of the reproductive success parameters are presented in Tables 4.17.

**Table 4.16**. Mean ± SD of reproductive success parameters recorded during 2021, 2022 and 2023. N indicates the number of nest boxes monitored in the different study areas. Dates are measured in days.

| Reproductive success | | Wood | | Urban | | Agri 1 | | Agri 2 | |
|---|---|---|---|---|---|---|---|---|---|
| | | Mean ± SD | N | Mean ± SD | N | Mean ± SD | N | Mean ± SD | N |
| **2021** | *Layind date* | 29.00±4.47 | 7 | 33.60±6.99 | 5 | 37.67±2.08 | 3 | 32.75±4.50 | 4 |
| | *Clutch size* | 7.14±1.07 | | 7.20±0.84 | | 9.00±2.00 | | 7.00±0.00 | |
| | *Hatching success* | 1.00±0.00 | | 1.00±0.00 | | 1.00±0.00 | | 0.96±0.08 | |
| | *Fledging success* | 0.94±0.11 | | 0.86±0.14 | | 0.97±0.06 | | 0.92±0.09 | |
| **2022** | *Layind date* | 32.27±2.37 | 11 | 33.14±2.48 | 7 | 36.00±4.66 | 8 | 37.33±3.39 | 10 |
| | *Clutch size* | 8.45±0.69 | | 7.00±1.53 | | 8.63±2.20 | | 7.00±1.63 | |
| | *Hatching success* | 0.90±0.30 | | 0.70±0.48 | | 0.66±0.44 | | 0.83±0.30 | |
| | *Fledging success* | 0.66±0.43 | | 0.53±0.51 | | 0.71±0.44 | | 0.81±0.34 | |
| **2023** | *Layind date* | 26.00±2.24 | 9 | 26.17±5.19 | 6 | 30.00±4.40 | 4 | 36.73±5.48 | 11 |
| | *Clutch size* | 8.44±0.88 | | 6.67±1.21 | | 9.50±1.00 | | 7.00±1.34 | |
| | *Hatching success* | 0.99±0.00 | | 0.78±0.40 | | 0.90±0.08 | | 0.86±0.11 | |
| | *Fledging success* | 0.61±0.42 | | 0.76±0.41 | | 1.00±0.00 | | 0.82±0.20 | |

**Table 4.17**. Outcomes of linear mixed models used to examine effects of each area on reproductive performances in great tit (*Parus major*) nestlings sampled in 2021, 2022 and 2023. *p< 0.05, indicate significant difference.

| Variable | Factor | Reference Level | Level | Coefficient Estimate ± SE | *t* | *p* |
|---|---|---|---|---|---|---|
| **Laying date** | *Area* | Wood | Urban | 1.641 ± 1.349 | 1.216 | 0.227 |
| | | Wood | Agri 1 | 5.008 ± 1.433 | 3.494 | 0.0008* |
| | | Wood | Agri 2 | 7.360 ± 1.252 | 5.879 | <0.0001* |
| | | Urban | Agri 1 | 3.366 ± 1.557 | 2.162 | 0.0337* |
| | | Urban | Agri 2 | 5.719 ± 1.391 | 4.111 | <0.0001* |
| | *Sampling year* | 2021 | 2022 | 1.638 ± 1.269 | 1.291 | 0.201 |
| | *Sampling year* | 2021 | 2023 | -2.791 ± 1.311 | -2.129 | 0.036* |
| **Clutch size** | *Area* | Wood | Urban | -1.161 ± 0.403 | -2.88 | 0.005* |
| | | Wood | Agri 1 | 0.808 ± 0.428 | 1.887 | 0.063 |
| | | Wood | Agri 2 | -1.152 ± 0.370 | -3.116 | 0.0026* |
| | | Urban | Agri 1 | 1.968 ± 0.465 | 4.233 | <0.0001* |
| | | Urban | Agri 2 | 0.009 ± 0.412 | 0.021 | 0.983 |
| | | Agri 1 | Agri 2 | -1.960 ± 0.436 | -4.497 | <0.0001* |
| | *Sampling year* | 2021 | 2022 | 0.330 ± 0.378 | 0.875 | 0.384 |
| | *Sampling year* | 2021 | 2023 | 0.405 ± 0.392 | 1.035 | 0.304 |
| **Hatching success** | *Area* | Wood | Urban | -0.150 ± 0.081 | -1.847 | 0.068 |
| | | Wood | Agri 1 | -0.142 ± 0.086 | -1.647 | 0.104 |
| | | Wood | Agri 2 | -0.082 ± 0.075 | -1.095 | 0.277 |
| | *Sampling year* | 2021 | 2022 | -0.200 ± 0.076 | -2.619 | 0.0106* |
| | *Sampling year* | 2021 | 2023 | -0.107 ± 0.079 | -1.349 | 0.181 |
| **Fledging success** | *Area* | Wood | Urban | -0.020 ± 0.103 | -0.195 | 0.846 |
| | | Wood | Agri 1 | 0.142 ± 0.109 | 1.296 | 0.199 |
| | | Wood | Agri 2 | 0.132 ± 0.095 | 1.398 | 0.166 |
| | *Sampling year* | 2021 | 2022 | -0.254 ± 0.097 | -2.626 | 0.010* |
| | *Sampling year* | 2021 | 2023 | -0.169 ± 0.100 | -1.69 | 0.095 |

*Laying date*

Regarding the laying date, the analysis of the collected data shows a statistically significant difference between 2021 and 2023; in 2023 the laying date is estimated a week before those of 2022 and 2021 (Table 4.17). A significant difference was also found among study areas, with great tit populations from wood and urban areas having an anticipation of the laying date compared to agricultural areas. The significant anticipation of laying dates in 2023 and in wood and urban areas is probably linked to climate change and the resulting rise of temperatures. As reported by Bodey et al. (2021) and Andreasson et al. (2023), avian species such as great tit can modify and adapt their phenology on the peak of food availability, which appears anticipated due to increasing spring temperatures. The climate change effect is emphasized in "thermophilic" zones, having a warmer microclimate, such as wood (Euganean Hills) and urban (Padoa center) areas of our study. In particular, the former has higher temperatures and particular geothermal characteristics due to its

volcanic origin (Kaltenrieder et al., 2010), and the latter represents a sort of heat island (Debbage and Shepherd, 2015). Therefore, it would be interesting in future studies to quantify the insect prey abundance of the great tit in the study areas with different temperatures in order to investigate the food availability and quality.

*Clutch size*

The clutch size was significantly reduced in urban and agri 2 areas with respect to wood and agri 1 sites. The clutch size reduction recorded in urban area is consistent with the study of De Satgé et al. (2019). These authors found a significant smaller clutch sizes in more urbanized areas and in parks of small towns compared to rural areas. Therefore, we can hypothesize that the clutch size reduction of urban area, together with more focused parental care on less nestlings, could be due to a sort of parents strategy to ensure an higher fledging success (Saulnier et al., 2023).

*Hatching and fledging successes*

We recorded a statistical difference in hatching and fledging successes between 2021 and 2022 and they were higher in 2022; this could be caused by the reduced data amount related to hatching and fledging success for the 2021 breading season (Table 4.17). Thus, further monitoring campaigns should be conducted in the coming years in order to obtain a better vision on these parameters.
No significant differences were found for hatching and fledging success at the areas level. This could be due to the fact that European populations of *P. major* are ubiquitous (Charmantier et al., 2017) and are able to adapt itself, thanks to parental strategies, to different and anthropized and not anthropized environments (Wawrzyniak et al., 2020).

## 4.5. Conclusions of great tit ecotoxicological investigations

This part of the thesis work allowed to acquire new knowledges on the ecotoxicological status of great tit populations from areas with different anthropic impacts (wood, urban and agricultural) by applying a multi-biomarker and complementary approach based on contaminant analysis, biomarker responses, behavioural traits analysis and breeding performances. This holistic approach is an effective tool for assessing sub-lethal effects at different levels of biological organization and for evaluating the state of the great tit populations exposed to different and multiple stressors.

We can draw the following specific conclusions:

- The assessment of heavy metal levels (cadmium, mercury, lead, and copper) on feathers showed that these compounds had the following trend in all the sampling areas, Cu > Pb > Hg > Cd. OCPs

evaluated in abandoned/unhatched eggs revealed a predominance of ∑DDTs followed by HxCB, ∑HCHs, PeCB, and ∑endosulfan (∑DDTs > HxCB > ∑HCHs > PeCB > ∑endosulfan). Lastly, PFOS and PFCAs were the most abundant compounds among the PFAS evaluated in abandoned/unhatched eggs;

- The urban area showed the highest levels of Pb and Hg followed by agri 2 area, which is 15 km away from the center of Padova. Pb and Hg levels could be due to high traffic, local industrial and commercial emissions. However, if compared to other studies, the analyzed heavy metals have not exceeded the threshold values. The urban area resulted again the most polluted site in relation to OPCs concentrations followed, by agri 1, wood, agri 2 areas (Urban > Agri 1 > Wood > Agri 2), with the greatest contribution of pp'-DDE. Finally, the agri 1 area showed the highest levels of PFOS, and the urban and wood areas the highest levels of the different PFCAs. PFCA levels found in the urban site can be due to proximity to the emission sources while the levels of PFCA in the wood site can be due to the capture of PFCA precursors by plants.
- The set of biomarkers was successfully applied and permitted to evaluate toxicological responses at molecular, biochemical, and cellular level. We found an effect of the sampling year, sampling date, and body mass on several biomarkers applied. In fact, TAS, complement system activity, respiratory burst, eosinophils abundance, and BChE and CaE activities were higher in nestlings sampled in 2022 than in 2021. On the other hand, monocyte number and DNA damage were lower in 2022. Younger nestlings showed lower DNA fragmentation values and higher nuclear abnormalities. Moreover, heavier animals showed a greater ability to react to environmental pressures by implementing their own oxidative and immune defense mechanisms, thus showing lower TAS levels, better complement system activity, and lower values of H/L ratio;
- Significant differences emerged in toxicological responses among great tit populations from wooded, urban and agricultural study areas. In urban area, great tit responded to environmental pressures with a reduction of antioxidants, as a sort of adaptation system to external stressors. In this area high physiological stress and generalized inflammation conditions with high H/L ratio values and eosinophils' abundance were highlighted. We also found genotoxicity effects measured through ENA assay. The observed chromosomal damage could be due to heavy metals, OCPs and also by other compounds typical of an urban environment such as PAHs. In agri 1 area the levels of non enzymatic antioxidants were the highest probably due to the presence of contaminants such as PFOS, OCPs and other pesticides with pro-oxidant capacity. In this area immunotoxicity effects were recorded with an impairment of the complement system activity;

contaminants can compromise innate immune response as well as other environmental multiple stressors. Agri 1 area was also affected by genotoxicity effects potentially induced by a mix of contaminants including pesticides, definitely used in the fields around the area that is heavily exploited for agricultural purposes, OCPs and PFOS, detected in the eggs. The wood area showed an accumulation of all porphyrin types (copro-, uro-, and protoporphyrins) that could result from the exposure to Particulate Matters (PMs) from Padua city, deposited in the area through atmospheric agent, and by other toxic compounds such as dioxins and PCBs. In wood area emerged immunotoxicity effects with an impairment of the complement system activity, fundamental for a proper innate immune response. The longPFCA concentration recorded in eggs from the wood site highlighted that this kind of contamination affects this area, and these substances might have acted on nestlings' immune function. The agri 2 area presented high number of heterophils that proliferate in circulation in response to infections and inflammation conditions and genotoxicity effects. Among different causes for these effects, we can consider also heavy metals (Pb and Hg) that we found in nestlings' feathers.

- Integrating the contaminant information and the overall biomarkers responses above, we found that urban area was the most contaminated site (presence of Pb, Hg, OCPs and longPFCA), indeed a modification of the oxidative status, induction of immune response, and genotoxicity effects emerged. The agri 1 area was also impacted by contamination (OCPs and PFOS found in eggs and probable presence of other pesticides due to the intensive agricultural activities) and we recorded an alteration of the oxidative status, impairment of the immune function and genotoxicity effects. In the wood site emerged the presence of longPFAC in eggs, but it is also conceivable the presence of PMs and other pollutants that induced the severe porphyrins accumulation and immunotoxicity effects recorded in this area. Finally, the agri 2 area showed the presence of heavy metals (Pb and Hg), genotoxicity effects and an immune response induction potentially also caused by parasites presence.
- The behavioural traits of great tit nestlings from the different study areas were evaluated, and an effect of the sampling year, sampling day and body mass on great tit behaviour was found. Nestlings sampled in 2022 and 2023 showed higher breath rates and prolonged catatonic state than in 2021 and individuals born later during the season resulted more agitated. Furthermore, nestlings with higher body mass were more agitated and presented a shorter catatonic state, highlighting an effect of the body mass on the behaviour of the animals. We found that environments act on some aspects of the great tits populations studied: individuals from wood

and urban area showed the highest breath rate. We found interesting interlinkages among biomarkers and behavioural traits, thus connection at different level of biological organization. Indeed, correlations among behavioural traits with genotoxicity and neurotoxicity biomarkers and H/L ratio were found. Individuals more agitated (high breath rate and agitation state) and with bold personality (short tonic immobility) had higher irreversible genotoxic effects. Moreover, agitated nestlings, probably more stressed, presented high activity of the BChE and high values of H/L ratio that is a recognized index of physiological stress.

- The reproductive period of great tits from the study areas was monitored from the eggs laying up to fledging. An effect of the sampling year on some reproductive success factors was found. Indeed, the laying date was anticipated in 2023 respect to 2021, probably due to an increase in the temperatures in 2023. Hatching and fledging successes were higher in 2022 than in 2021, and this could be due to several factors, such as availability and quality of the food, parasites presence, favorable environmental conditions.
- Significant differences of some reproductive success parameters emerged among study areas. Great tit populations from wood and urban areas showed an anticipation of the laying date compared to agricultural areas. The anticipated laying dates in these sites could be related to climate change and the resulting rise of temperatures. Wood and urban areas of our study are "thermophilic" zones in which climate change effect can be emphasized. In urban and agri 2 areas a reduction of the clutch size emerged, but this difference did not have an effect on hatching and fledging success. Clutch size reduction is probably linked to a sort of parents' strategy to counteract multiple environmental stressors and to ensure a higher fledging success.
- A complete picture of the toxicological status of great tit populations was obtained by integrating biomarkers responses with contaminant analysis data and information on behavioral traits and reproduction success parameters. This study allowed to understand that the populations of urban and agri1 areas were those with the most significant impacts resulting from contamination and other possible pressures typical of anthropized environments. Considering the population of each study area, we found that nestlings of the urban area showed sub-lethal effects at the molecular/biochemical and cellular level. At the individual level, we found an environmental effect on nestlings' behaviour while no effects at population level such as reproductive success were found. In the wood area, hypothetically less impacted, emerged sub-lethal effects at the lower biological level (porphyrins accumulation and alteration of immune function). However, we did not record any effects at the higher biological level. Finally, in the great tit population

from agri 2 area emerged DNA alterations and induction of immune response. This population showed a behaviour alteration (nestlings more agitated) but no effects at the population level.

- We can conclude that the studied environments, characterized by the presence of different contaminant classes, differently impacted the health status of great tits populations, determining different biomarker and behaviour responses that in some cases showed an interconnection. Despitesub-lethal effects found at individual level, it would appear that the great tits have adopted strategies to avoid population effects, keeping a reproductive success (hatching and fledging) almost unchanged among the different environments.

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# CHAPTER 5
# FINAL CONCLUSIONS

# CHAPTER 5

## FINAL CONCLUSIONS

Birds play a crucial role within global ecosystems, fulfilling vital tasks. However, change of natural environment caused by the growth of cities, intensified agriculture, and expanding industries have a profound impact on bird populations exposing them to multiple stressors. In this study we:

- successfully conducted an assessment of the toxicological status of two bird species (*Falco tinnunculus* and *Parus major*) using a multi-biomarker and complementary approach based on contaminant analysis, biomarkers responses and behavioural and breeding performances. The multi-biomarker and complementary approach demonstrated to be a valid tool to investigate the complex condition to which birds are exposed and thus to determine biological and toxicological responses by these organisms.
- investigated the influence of environmental changes and multi-stressors on the physiological state of the two bird species.
- successfully developed genotoxicity and immunotoxicity biomarkers. Their development permitted to extend the biomarkers battery that can be used *in vivo* and *in vitro* studies on Common Kestrel, but also in other bird species enabling the measurement of fundamental endpoints for evaluating the health status of the avian populations. Non-invasive biomarkers have been validated on blood for future *in vitro* studies. The laboratory studies confirmed the importance of the *in vitro* approach to investigate the ecotoxicology of avian species. Indeed, this study demonstrated the usefulness of biomarkers for *in vitro* studies, demonstrating that they are sensitive tools for evaluating the toxicity of contaminants, such as pesticides. *In vitro* studies contributed to obtain important information on the genotoxicity for non-targeted species of two widely used commercial formulates, a fungicide and an herbicide. In a broader view, what is even more important is that the *in vitro* approach developed in this thesis has shown its effectiveness in testing the toxicity of commercial pesticide formulations instead of only active principles. This approach can be considered a valuable tool in regulatory activities to monitor the safety and toxicity of existing products and during the development of new ones.
- theorized a multi-tier approach, combining data of contaminant analysis, biomarker responses, behavioural traits and reproductive success. Specifically, we developed instruments that integrate chemical analysis with other endpoints for assessing effects at different levels of biological organization, from molecular/biochemical or sub-individual levels, evaluating oxidative stress,

- porphyrin levels, effects on immune system, neurotoxic and genotoxic effects, to individual level, investigating nestlings' personality and agitation state, up to population level, evaluating reproductive success parameters such as hatching and fledging success.
- successfully applied the multi-biomarkers and complementary approach on wild Common Kestrel and Great Tit populations inhabiting areas characterized by different anthropic pressures. The developed approach enables to acquire interesting information on the impact of the different environments and thus the various stressors on the studied bird populations. Specifically, in the field monitoring of Common Kestrel populations physiology changes, genotoxic and neurotoxic effects in kestrels from the diverse environments emerged. This shows the effectiveness of the multi-biomarker and complementary method in identifying different biological and toxicological responses due to environmental stressors that differ in typology or intensity across habitats. The multi-biomarkers and complementary approach applied on great tit populations allowed to get a complete picture of the toxicological status of great tit populations by integrating biomarkers responses with contaminant analysis data and information on behavioral traits and reproduction success parameters. We found that the great tit populations of urban and agri 1 areas were those with the most significant impacts resulting from environmental pressures. In addition, thanks to this holistic approach, it was also possible to identify sub-lethal effects in individuals from the other areas, showing the importance of evaluating different endpoints during a monitoring study. Although biomarker alterations emerged, no evident effects at the population level were found. However, it must be taken into account that biomarker alterations represent early warning signals of possible long-term effects for individuals and populations, therefore a longer-term monitoring is needed.
- The complementary approach applied in this study could be applied to monitor other birds' species considered as targets. Furthermore, this approach can be proposed also at managerial and regulatory level for the conservation and protection of wild and endangered bird species.

# SUPPLEMENTARY MATERIAL

**SUPPLEMENTARY MATERIAL**

# State of the art, gaps and future perspectives on common kestrel ecotoxicology

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**Table A1: Number of studies that met the criteria for inclusion in this review, divided by heavy metals investigated. Thirteen of the 19 papers on heavy metals measured more the one element.**

| Heavy metals | Number of publications |
|---|---|
| Aluminum (Al) | 3 |
| Antimony (Sb) | 2 |
| Arsenic (As) | 3 |
| Beryllium (Be) | 1 |
| Bismuth (Bi) | 2 |
| Cadmium (Cd) | 12 |
| Chrome (Cr) | 6 |
| Cobalt (Co) | 3 |
| Copper (Cu) | 8 |
| Iron (Fe) | 4 |
| Lead (Pb) | 13 |
| Manganese (Mn) | 5 |
| Mercury (Hg) | 9 |
| Molybdenum (Mo) | 2 |
| Nickel (Ni) | 5 |
| Selenium (Se) | 3 |
| Titanium (TI) | 1 |
| Vanadium (V) | 3 |
| Zinc (Zn) | 9 |

**Table A2: Results of articles on accumulation. The abbreviation *ns* means "not specified", *nd* indicate "not detected" and asterisk (*) refers to indicative results extrapolated from the graphs. The symbol "+" indicates the presence of the contaminant, while the symbol "-" indicates the absence of it.**

| Life stage | Sex | Study type | Biological material | Contaminant | Sample size | Results (mean±sd; mean; min-max) | | Unit of measurement | Reference, year |
|---|---|---|---|---|---|---|---|---|---|
| ns | ns | Field | Feathers | Hg | 53 | + | **0.0 - 1.51** | mg/Kg d.w. | Ma et al. 2021 |
| ns | ns | Field | Muscle | PCDD/Fs | 25 | + | **22.7 - 5280** | pg/g l.w. | Zhang et al. 2021 |
| Juvenile | F | | | | | + | **100 - 5000 *** | | |
| | M | | | | | + | **100 - 500*** | | |
| Adult | F | | | | | + | **25 - 3000*** | | |
| | M | | | | | + | **75- 7500 *** | | |
| ns | ns | | | Dioxin-like PCBs | | + | **4.91 - 1560** | | |
| Juvenile | F | | | | | + | **9 -700*** | | |
| | M | | | | | + | **9 '- 40*** | | |
| Adult | F | | | | | + | **8 - 100*** | | |
| | M | | | | | + | **20 - 100*** | | |
| ns | ns | | | PCBs | | + | **33.1 - 7950** | | |
| Juvenile | F | | | | | + | **75 - 7500*** | | |
| | M | | | | | + | **100-500*** | | |
| Adult | F | | | | | + | **50 - 800*** | | |
| | M | | | | | + | **200 - 9000*** | | |
| ns | ns | Field | Liver | Al | 200 | + | **27.390 ± 23.475** | mg/Kg d.w. | Rodríguez-Álvarez et al. 2021 |
| ns | ns | Field | Liver | Cd | | + | **0.040 ± 0.062** | mg/Kg d.w. | |
| ns | ns | Field | Liver | Co | | + | **0.001 ± 0.012** | mg/Kg d.w. | |
| ns | ns | Field | Liver | Cr | | + | **0.015 ± 0.089** | mg/Kg d.w. | |
| ns | ns | Field | Liver | Cu | | + | **9.573 ± 7.284** | mg/Kg d.w. | |
| ns | ns | Field | Liver | Fe | | + | **181.635 ± 169.217** | mg/Kg d.w. | |
| ns | ns | Field | Liver | Mn | | + | **2.698 ± 1.240** | mg/Kg d.w. | |
| ns | ns | Field | Liver | Mo | | + | **0.352 ± 0.194** | mg/Kg d.w. | |
| ns | ns | Field | Liver | Ni | | + | **0.202 ± 0.278** | mg/Kg d.w. | |
| ns | ns | Field | Liver | Pb | | + | **0.187 ± 0.133** | mg/Kg d.w. | |
| ns | ns | Field | Liver | Zn | | + | **27.602 ± 9.804** | mg/Kg d.w. | |
| ns | ns | Field | Liver | V | | + | **0.279 ± 0.457** | mg/Kg d.w. | |
| ns | ns | Field | Liver | Li | | + | **8.470 ± 5.702** | mg/Kg d.w. | |
| ns | ns | Field | Liver | Sr | | + | **2.976 ± 2.955** | mg/Kg d.w. | |
| ns | ns | Field | Liver | B | | + | **0.632 ± 0.511** | mg/Kg d.w. | |
| ns | ns | Field | Liver | Ba | | + | **1.238 ± 0.875** | mg/Kg d.w. | |
| ns | ns | Field | Muscle | Al | | + | **18.139 ± 24.096** | mg/Kg d.w. | |
| ns | ns | Field | Muscle | Cd | | + | **0.003 ± 0.015** | mg/Kg d.w. | |
| ns | ns | Field | Muscle | Co | | + | **0.001 ± 0.007** | mg/Kg d.w. | |
| ns | ns | Field | Muscle | Cr | | + | **0.042 ± 0.170** | mg/Kg d.w. | |
| ns | ns | Field | Muscle | Cu | | + | **8.179 ± 3.613** | mg/Kg d.w. | |

| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| ns | ns | Field | Muscle | F | | + | **84.921 ± 42.298** | mg/Kg d.w. | |
| ns | ns | Field | Muscle | Mn | | + | **0.760 ± 0.406** | mg/Kg d.w. | |
| ns | ns | Field | Muscle | Mo | | + | **0.013 ± 0.072** | mg/Kg d.w. | |
| ns | ns | Field | Muscle | Ni | | + | **0.126 ± 0.293** | mg/Kg d.w. | |
| ns | ns | Field | Muscle | Pb | | + | **0.148 ± 0.095** | mg/Kg d.w. | |
| ns | ns | Field | Muscle | Zn | | + | **20.463 ± 8.324** | mg/Kg d.w. | |
| ns | ns | Field | Muscle | V | | + | **0.370 ± 0.471** | mg/Kg d.w. | |
| ns | ns | Field | Muscle | Li | | + | **5.827 ± 5.282** | mg/Kg d.w. | |
| ns | ns | Field | Muscle | Sr | | + | **3.434 ± 5.659** | mg/Kg d.w. | |
| ns | ns | Field | Muscle | B | | + | **0.339 ± 0.401** | mg/Kg d.w. | |
| ns | ns | Field | Muscle | Ba | | + | **1.289 ± 1.466** | mg/Kg d.w. | |
| ns | ns | Field | Feathers | Al | | + | **298.593 ± 139.768** | mg/Kg d.w. | |
| ns | ns | Field | Feathers | Cd | | + | **0.061 ± 0.076** | mg/Kg d.w. | |
| ns | ns | Field | Feathers | Co | | + | **0.260 ± 0.776** | mg/Kg d.w. | |
| ns | ns | Field | Feathers | Cr | | + | **0.823 ± 2.620** | mg/Kg d.w. | |
| ns | ns | Field | Feathers | Cu | | + | **11.817 ± 7.774** | mg/Kg d.w. | |
| ns | ns | Field | Feathers | Fe | | + | **223.714 ± 319.090** | mg/Kg d.w. | |
| ns | ns | Field | Feathers | Mn | | + | **13.980 ± 17.672** | mg/Kg d.w. | |
| ns | ns | Field | Feathers | Mo | | + | **0.902 ± 6.081** | mg/Kg d.w. | |
| ns | ns | Field | Feathers | Ni | | + | **0.781 ± 1.829** | mg/Kg d.w. | |
| ns | ns | Field | Feathers | Pb | | + | **4.353 ± 20.645** | mg/Kg d.w. | |
| ns | ns | Field | Feathers | Zn | | + | **198.469 ± 520.795** | mg/Kg d.w. | |
| ns | ns | Field | Feathers | V | | + | **2.679 ± 10.819** | mg/Kg d.w. | |
| ns | ns | Field | Feathers | Li | | + | **4.411 ± 8.209** | mg/Kg d.w. | |
| ns | ns | Field | Feathers | Sr | | + | **9.573 ± 9.972** | mg/Kg d.w. | |
| ns | ns | Field | Feathers | B | | + | **3.955 ± 4.061** | mg/Kg d.w. | |
| ns | ns | Field | Feathers | Ba | | + | **7.663 ± 6.519** | mg/Kg d.w. | |
| ns | ns | Field | Liver | Brodifacoum | 14 | + | **1.4 – 1174** | ng/g | Rial-Berriel et al. 2021 |
| ns | ns | Field | Liver | Bromadiolone | | + | **2.6 – 4840.3** | ng/g | |
| ns | ns | Field | Liver | Difenacoum | | + | **1.2 – 33.4** | ng/g | |
| ns | ns | Field | Liver | Flocoumafen | | + | **0.5 – 10.8** | ng/g | |
| ns | ns | Field | Liver | Difethialone | | + | **2.4** | ng/g | |
| Adult | ns | Field | Feathers | Al | 37 | + | **87.1 - 3524** | mg/Kg d.w. | Manzano et al. 2021 |
| Adult | ns | Field | Feathers | Be | | - | | | |
| Adult | ns | Field | Feathers | Bi | | - | | | |
| Adult | ns | Field | Feathers | Cd | | + | **<LOD - 1.002** | mg/Kg d.w. | |
| Adult | ns | Field | Feathers | Co | | - | | | |
| Adult | ns | Field | Feathers | Cr | | + | **0.359 - 6.55** | mg/Kg d.w. | |
| Adult | ns | Field | Feathers | Cu | | + | **4.46 - 43.3** | mg/Kg d.w. | |
| Adult | ns | Field | Feathers | Fe | | + | **72.4 - 2424** | mg/Kg d.w. | |
| Adult | ns | Field | Feathers | Mn | | + | **1.10 - 21.1** | mg/Kg d.w. | |
| Adult | ns | Field | Feathers | Mo | | + | **0.047 - 1.27** | mg/Kg d.w. | |
| Adult | ns | Field | Feathers | Ni | | + | **0.230 - 2.60** | mg/Kg d.w. | |
| Adult | ns | Field | Feathers | Pb | | + | **0.421 - 7.65** | mg/Kg d.w. | |

| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| Adult | ns | Field | Feathers | Se | | - | | mg/Kg d.w. | |
| Adult | ns | Field | Feathers | Tl | | - | | mg/Kg d.w. | |
| Adult | ns | Field | Feathers | Zn | | + | **5.54 - 72.5** | mg/Kg d.w. | |
| Adult | ns | Field | Feathers | V | | + | **<LOD - 6.35** | mg/Kg d.w. | |
| Adult | ns | Field | Feathers | Ca | | + | **50.6 - 567** | mg/Kg d.w. | |
| Adult | ns | Field | Feathers | K | | + | **11.2 - 1233** | mg/Kg d.w. | |
| Adult | ns | Field | Feathers | Li | | + | **<LOD - 3.26** | mg/Kg d.w. | |
| Adult | ns | Field | Feathers | Mg | | + | **20.8 - 888** | mg/Kg d.w. | |
| Adult | ns | Field | Feathers | Na | | - | | | |
| Adult | ns | Field | Feathers | Rb | | + | **0.435 - 4.36** | mg/Kg d.w. | |
| Adult | ns | Field | Feathers | Sb | | + | **<LOD - 2.23** | mg/Kg d.w. | |
| Adult | ns | Field | Feathers | Sr | | + | **0.164 - 3.82** | mg/Kg d.w. | |
| Adult | ns | Field | Feathers | Ti | | + | **2.97 - 86.8** | mg/Kg d.w. | |
| Adult | ns | Field | Feathers | P | | + | **51.4 - 367** | mg/Kg d.w. | |
| Adult | ns | Field | Feathers | S | | + | **15.896 - 29.258** | mg/Kg d.w. | |
| Eggs | ns | Field | Egg | PCBs | 40 | + | **30.4 - 2973.1** | ng/g d.w. | Buck et al. 2020 |
| Eggs | ns | Field | Egg | HCB | | + | **0.1 - 10.5** | ng/g d.w. | |
| Eggs | ns | Field | Egg | HCH | | + | **0.00 - 113.33** | ng/g d.w. | |
| Eggs | ns | Field | Egg | DDTs | | + | **785.6 - 50532.2** | ng/g d.w. | |
| ns | ns | Field | Blood | PAHs | 112 | + | **0.19 - 2.67 *** | ng/ml | Rial-Berriel et al. 2020 |
| ns | ns | Field | Blood | PCBs | | + | **0.09 - 6.88** | ng/ml | |
| ns | ns | Field | Blood | PBDEs | | + | **0.23** | ng/ml | |
| ns | ns | Field | Blood | DDTs, HCH, Coumaphos, Metaflumizone | | + | **0.09 - 5.95** | ng/ml | |
| ns | ns | Field | Blood | Brodifacoum, Bromadiolone, Coumachlor, Coumatetralyl, Difenacoum, Defethialone | | + | **0.3 - 32.73** | ng/ml | |
| ns | ns | Field | Blood | Hexachlorobenzene, 2-Phenylphenol, Benalaxyl, Metrafenone | | + | **0.1 - 5.49** | ng/ml | |
| ns | ns | Field | Blood | Atrazine, Simazine | | + | **0.1 - 0.29** | ng/ml | |
| ns | ns | Field | Blood | Albenzadole, Enrofloxacin, Fenbendazole, Flumequine, Levamisole, Mebendazole, Sulfachloropiridacine, Sulfadiacine, Sulfapyridine | | + | **0.1 - 1.2** | ng/ml | |
| Adult | M | In vivo | Liver | Bromadiolone | 12 | + | **756 - 8848** | ng/g d.w. | Valverde et al. 2020 |
| Adult | M | In vivo | Blood | Bromadiolone | | + | **57 - 135** | ng/g w.w. | |
| Adult | F | In vivo | Liver | Bromadiolone | | + | **2892 - 3000** | ng/g d.w. | |
| Adult | F | In vivo | Blood | Bromadiolone | | + | **45 - 47** | ng/g w.w. | |
| ns | ns | Field | Liver | Pb | 39 | + | **0.03 ± 0.04 *** | ppm | Kanstrup et al. 2019 |
| ns | ns | Field | Liver | Cd | | + | **0.05 ± 0.04 *** | ppm | |
| ns | ns | Field | Liver | Hg | | + | **0,16 ± 0,17 *** | ppm | |
| ns | ns | Field | Liver | Bi | | + | **3,13 ± 11,92 *** | ppm | |
| ns | ns | Field | Liver | Se | | + | **1,08 ± 0,22 *** | ppm | |
| ns | ns | Field | Stomach contents | ns | 0 | - | | | Bang et al. 2019 |
| ns | ns | Field | Liver | Cd | 7 | + | **0 - 0.4*** | mg/Kg d.w. | Soliman et al. 2019 |
| ns | ns | Field | Liver | Pb | | + | **2-5*** | mg/Kg d.w. | |
| ns | ns | Field | Liver | Cu | | + | **30 - 35*** | mg/Kg d.w. | |
| ns | ns | Field | Liver | Zn | | + | **130 - 150*** | mg/Kg d.w. | |
| Adult | ns | Field | Feathers | As | 18 | + | **0.30 ± 0.30** | mg/Kg | Gruz et al. 2019 |

| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| Adult | ns | Field | Feathers | Cd | | + | **0.21 ± 0.19** | mg/Kg | |
| Adult | ns | Field | Feathers | Hg | | + | **0.46 ± 0.29** | mg/Kg | |
| Adult | ns | Field | Feathers | Pb | | + | **1.85 ± 1.24** | mg/Kg | |
| Juvenile | ns | Field | Feathers | As | | + | **0.30 ± 0.16** | mg/Kg | |
| Juvenile | ns | Field | Feathers | Cd | | + | **0.20 ± 0.18** | mg/Kg | |
| Juvenile | ns | Field | Feathers | Hg | | + | **0.75 ± 0.36** | mg/Kg | |
| Juvenile | ns | Field | Feathers | Pb | | + | **2.48 ± 2.02** | mg/Kg | |
| ns | M | Field | Feathers | As | | + | **0.20 ± 0.00** | mg/Kg | |
| ns | M | Field | Feathers | Cd | | + | **0.13 ± 0.10** | mg/Kg | |
| ns | M | Field | Feathers | Hg | | + | **0.64 ± 0.37** | mg/Kg | |
| ns | M | Field | Feathers | Pb | | + | **2.02 ± 1.30** | mg/Kg | |
| ns | F | Field | Feathers | As | | + | **0.36 ± 0.32** | mg/Kg | |
| ns | F | Field | Feathers | Cd | | + | **0.25 ± 0.20** | mg/Kg | |
| ns | F | Field | Feathers | Hg | | + | **0.56 ± 0.37** | mg/Kg | |
| ns | F | Field | Feathers | Pb | | + | **2.50 ± 1.81** | mg/Kg | |
| Adult | ns | Field | Liver | PCBs | 4 | + | **23.5–1350** | ng/g lipid w. | Barghi et al. 2018 |
| Adult | ns | Field | Liver | DDTs | | + | **9.15 – 220** | ng/g lipid w. | |
| Adult | ns | Field | Liver | CHLs | | + | **50 – 421** | ng/g lipid w. | |
| Adult | ns | Field | Liver | CBz | | + | **0.15 – 25.7** | ng/g lipid w. | |
| Adult | ns | Field | Liver | PFASs | | + | **25.9 – 652** | ng/g lipid w. | |
| Adult | ns | Field | Feathers | Hg | 5 | + | **0.054 ± 0.007*** | mg/Kg d.w. | Durmuş et al. 2018 |
| ns | ns | Field | Feathers | PBDEs | 17 | + | **1.5 – 191** | ng/g d.w. | Yin et al. 2018 |
| ns | ns | Field | Muscle | PBDEs | | + | **83 – 1.43×10^4** | ng/g lipid w. | |
| Nestling | ns | Field | Blood | Bromadiolone | 112 | + | **0.25 ± 0.02 *** | ng/mL | Martínez-Padilla et al. 2017 |
| ns | ns | Field | Blood | Pb | 10 | + | **ns** | µg/L | Berny et al. 2017 |
| Adult | M | Hystorical col. | Feathers | Hg | 16 | + | **0.4 - 4.5** | mg/Kg d.w. | Movalli et al. 2017 |
| Adult | M | Hystorical col. | Feathers | Cd | | + | **> 1** | mg/Kg d.w. | |
| Adult | M | Hystorical col. | Feathers | Zn | | + | **122 - 423** | mg/Kg d.w. | |
| Adult | M | Hystorical col. | Feathers | Se | | + | **≤2** | mg/Kg d.w. | |
| Adult | M | Hystorical col. | Feathers | Al | | + | **100 - 800** | mg/Kg d.w. | |
| Adult | M | Hystorical col. | Feathers | As | | + | **14.4 - 596.9** | mg/Kg d.w. | |
| Adult | M | Hystorical col. | Feathers | Cr | | + | **3-20.65** | mg/Kg d.w. | |
| Adult | M | Hystorical col. | Feathers | Sb | | + | **0 - 11.06** | mg/Kg d.w. | |
| Adult | M | Hystorical col. | Feathers | V | | + | **0.086 - 3.3** | mg/Kg d.w. | |
| Adult | M | Hystorical col. | Feathers | Pd | | + | **0 - 1.7** | mg/Kg d.w. | |
| Adult | M | Hystorical col. | Feathers | Pt | | + | **0 - 0.65** | mg/Kg d.w. | |
| Adult | M | Hystorical col. | Feathers | Rb | | + | **0 - 1.975** | mg/Kg d.w. | |
| Adult | M | Hystorical col. | Feathers | Cl | | + | **500 - 2760** | mg/Kg d.w. | |
| Adult | M | Hystorical col. | Feathers | Br | | + | **3.6 - 160** | mg/Kg d.w. | |
| Eggs | ns | Field | Egg | BP1 | 10 | + | **27.9 - 53.5** | ng/g d.w. | Molins-Delgado et al. 2017 |
| Eggs | ns | Field | Egg | BP3 | | + | **18.3 - 35.2** | ng/g d.w. | |
| Eggs | ns | Field | Egg | 4HB | | + | **20 - 1200** | ng/g d.w. | |
| Eggs | ns | Field | Egg | 4DHB | | + | **<MLOQ - 132** | ng/g d.w. | |
| Eggs | ns | Field | Egg | ODPABA | | + | **<MLOQ - <MLOQ** | ng/g d.w. | |

| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| Eggs | ns | Field | Egg | OC | | + | **<MLOQ - <MLOQ** | ng/g d.w. | |
| Eggs | ns | Field | Egg | UVP | | + | **0.4 - 3.0** | ng/g d.w. | |
| ns | ns | Field | Feathers | PBDEs | 4 | + | **0.1 - 1.0** | ng/g d.w. | Abbasi et al. 2017 |
| ns | ns | Field | Feathers | HBCDDs | | + | **0.2 - 3.4** | ng/g d.w. | |
| ns | ns | Field | Feathers | BTBPE | | - | **<LOQ (0.3)*** | | |
| ns | ns | Field | Feathers | BEHTBP | | - | **<LOQ (0.0)*** | | |
| ns | ns | Field | Feathers | EH-TBB | | - | **<LOQ (0.0)*** | | |
| ns | ns | Field | Gastric ct | Monocrotophos | 2 | - | | | Kim S. et al. 2016 |
| ns | ns | Field | Gastric ct | Phosphamidon | | - | | | |
| ns | ns | Field | Gastric ct | Diazinon | | - | | | |
| ns | ns | Field | Gastric ct | EPN | | - | | | |
| ns | ns | Field | Gastric ct | Fenitrothion | | - | | | |
| ns | ns | Field | Gastric ct | Fenthion | | - | | | |
| ns | ns | Field | Gastric ct | Methidathion | | - | | | |
| ns | ns | Field | Gastric ct | Parathion | | - | | | |
| ns | ns | Field | Gastric ct | Edifenphos | | - | | | |
| ns | ns | Field | Gastric ct | Phorate | | - | | | |
| ns | ns | Field | Gastric ct | Endosulfan | | - | | | |
| ns | ns | Field | Gastric ct | Cabofuran | | - | | | |
| ns | ns | Field | Gastric ct | Methomyl | | - | | | |
| Adult | ns | Field | Liver | PBDEs | 4 | + | **56.5 - 173** | ng/g lipid w. | Jin et al. 2016 |
| Adult | ns | Field | Liver | BEHTBP | | + | **2.88 - 110** | ng/g lipid w. | |
| Adult | ns | Field | Liver | DBDPE | | + | **<7.50 - 93.3** | ng/g lipid w. | |
| Adult | ns | Field | Liver | BTBPE | | + | **<0.30** | ng/g lipid w. | |
| Adult | ns | Field | Liver | DP | | + | **<0.04 - 3052** | ng/g lipid w. | |
| Adult | ns | Field | Liver | Pb | 3 | + | **0.652 - 0.787** | mg/Kg d.w. | Kitowski et al. 2016 |
| Adult | ns | Field | Liver | Cd | | + | **0.156 - 0.410** | mg/Kg d.w. | |
| Adult | ns | Field | Liver | Ni | | + | **0.321 - 0.364** | mg/Kg d.w. | |
| Adult | ns | Field | Liver | Cr | | + | **0.597 - 0.662** | mg/Kg d.w. | |
| Adult | ns | Field | Liver | Hg | | + | **0.069 - 1.659** | mg/Kg d.w. | |
| Adult | ns | Field | Liver | Fe | 25 | + | **2051 ± 1395** | µg/g d.w. | Kim J. et al. 2016 |
| Adult | ns | Field | Liver | Zn | | + | **132 ± 60.4** | µg/g d.w. | |
| Adult | ns | Field | Liver | Mn | | + | **16.0 ± 8.09** | µg/g d.w. | |
| Adult | ns | Field | Liver | Cu | | + | **45.1 ± 23.0** | µg/g d.w. | |
| Adult | ns | Field | Liver | Pb | | + | **11.7 ± 6.13** | µg/g d.w. | |
| Adult | ns | Field | Liver | Cd | | + | **1.87 ± 1.08** | µg/g d.w. | |
| Juvenile | ns | Field | Liver | Fe | | + | **769 ± 271** | µg/g d.w. | |
| Juvenile | ns | Field | Liver | Zn | | + | **102 ± 45.0** | µg/g d.w. | |
| Juvenile | ns | Field | Liver | Mn | | + | **15.4 ± 5.93** | µg/g d.w. | |
| Juvenile | ns | Field | Liver | Cu | | + | **23.4 ± 6.61** | µg/g d.w. | |
| Juvenile | ns | Field | Liver | Pb | | + | **3.48 ± 1.46** | µg/g d.w. | |
| Juvenile | ns | Field | Liver | Cd | | + | **0.78 ± 0.36** | µg/g d.w. | |
| Embryo | ns | Field | Egg | PFASs | 40 | + | **0.08 - 20.1** | ng/g | Eriksson et al. 2016 |
| ns | ns | Field | Kidney | Hg | 2 | + | **0.336 - 1.743** | mg/Kg d.w. | Kitowski et al. 2015 |

| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| Juvenile | ns | Field | Muscle | Hg | 3 | + | **0.001 - 0.041** | mg/Kg f.w. | Żarski et al. 2015 |
| Juvenile | ns | Field | Muscle | Hg | | + | **0.000 - 0.030** | mg/Kg f.w. | |
| Juvenile | ns | Field | Heart | Hg | | + | **0.001 - 0.095** | mg/Kg f.w. | |
| Juvenile | ns | Field | Bone | Hg | | + | **0.000 - 0.004** | mg/Kg f.w. | |
| Juvenile | ns | Field | Intestines | Hg | | + | **0.000 - 0.094** | mg/Kg f.w. | |
| Juvenile | ns | Field | Liver | Hg | | + | **0.001 - 0.094** | mg/Kg f.w. | |
| Juvenile | ns | Field | Kidney | Hg | | + | **0.002 - 0.022** | mg/Kg f.w. | |
| Juvenile | ns | Field | Lungs | Hg | | + | **0.001 - 0.056** | mg/Kg f.w. | |
| Juvenile | ns | Field | Gizzard | Hg | | + | **0.001 - 0.012** | mg/Kg f.w. | |
| Juvenile | ns | Field | Brain | Hg | | + | **0.000 - 0.025** | mg/Kg f.w. | |
| Juvenile | ns | Field | Stomach mucosus | Hg | | - | | | |
| ns | ns | Field | Feathers | Pb | 4 | + | **2.21 - 3.51** | µg/g | Abbasi et al. 2015 |
| ns | ns | Field | Feathers | Cd | | + | **0.80 - 1.03** | µg/g | |
| ns | ns | Field | Feathers | Cr | | + | **1.45 - 2.14** | µg/g | |
| ns | ns | Field | Feathers | Ni | | + | **1.12 - 2.15** | µg/g | |
| ns | ns | Field | Feathers | Co | | + | **0.35 - 1.89** | µg/g | |
| ns | ns | Field | Feathers | Cu | | + | **3.95 - 5.21** | µg/g | |
| ns | ns | Field | Feathers | Fe | | + | **120.54 - 136.20** | µg/g | |
| ns | ns | Field | Feathers | Mn | | + | **1.49 - 2.89** | µg/g | |
| ns | ns | Field | Feathers | Zn | | + | **83.26 -113.56** | µg/g | |
| ns | ns | Field | Liver | PCBs | 16 | + | **160** | pg/g lipid | Pereira et al. 2014 |
| ns | ns | Field | Liver | Dieldrin, Dicofol, DDTs, HCB, HCH | 20 | + | **692.9 – 10.036,6** | ng/g w.w. | Luzardo et al. 2014 |
| ns | ns | Field | Liver | PCBs | | + | **4.2 – 95.8** | ng/g w.w. | |
| ns | ns | Field | Liver | PAHs | | + | **138.2 – 4356.6** | ng/g w.w. | |
| ns | ns | Field | Liver | Bromadiolone | 21 | + | **8 - 151.4** | ng/g w.w. | Ruiz-Suárez et al. 2014 |
| ns | ns | Field | Liver | Brodifacoum | | + | **-14.7 - 129.5** | ng/g w.w. | |
| ns | ns | Field | Liver | Difenacoum | | + | **-6.2 - 22.6** | ng/g w.w. | |
| ns | ns | Field | Liver | Chlorophacinone | | + | **-0.6 - 1.8** | ng/g w.w. | |
| ns | ns | Field | Liver | Bromadiolone, Brodifacoum, Difenacoum | 22 | + | **0.173 ± 0.082** | mg/Kg | Hughes et al. 2013 |
| ns | ns | Field | Liver | Difethialone, Flocoumafen | | - | | | |
| ns | ns | Field | Liver | DDTs | 23 | + | **120 - 110.000** | ng/g lipid w. | Yu et al. 2013 |
| ns | ns | Field | Liver | PCBs | | + | **44 - 17.000** | ng/g lipid w. | |
| ns | ns | Field | Liver | PBDEs | | + | **120 - 8500** | ng/g lipid w. | |
| ns | ns | Field | Liver | HBCDs | | + | **<MDL - 260** | ng/g lipid w. | |
| ns | ns | Field | Liver | DP | | + | **<MDL - 60** | ng/g lipid w. | |
| Adult | ns | Field | Feathers | Cd | 1 | + | **5.2** | µg/g | Nighat et al. 2013 |
| Adult | ns | Field | Feathers | Zn | | + | **289.3** | µg/g | |
| Adult | ns | Field | Feathers | Ni | | + | **500** | µg/g | |
| Adult | ns | Field | Feathers | Pb | | + | **62.66** | µg/g | |
| Adult | ns | Field | Feathers | Cu | | - | **nd** | | |
| Adult | ns | Field | Feathers | As | | + | **44.55** | µg/g | |
| ns | ns | Field | Liver | Zn | 1 | + | **48.70** | mg/Kg d.w. | Komosa et al. 2012 |
| ns | ns | Field | Liver | Cu | | + | **13.86** | mg/Kg d.w. | |
| ns | ns | Field | Liver | Mn | | + | **5.04** | mg/Kg d.w. | |

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|---|---|---|---|---|---|---|---|---|---|
| ns | ns | Field | Liver | Cd | | + | **0.24** | mg/Kg d.w. | |
| ns | ns | Field | Liver | Pb | | + | **1.14** | mg/Kg d.w. | |
| ns | ns | Field | Liver | Cr | | + | **1.77** | mg/Kg d.w. | |
| ns | ns | Field | Liver | Brodifacoum | 38 | + | **2** | ng/g w.w. | Christensen et al. 2012 |
| ns | ns | Field | Liver | Bromadiolone | | - | | | |
| ns | ns | Field | Liver | Coumatetralyl | | - | | | |
| ns | ns | Field | Liver | Difenacoum | | + | **6.5** | ng/g w.w. | |
| ns | ns | Field | Liver | Flocoumafen | | - | | | |
| ns | ns | Field | Liver | DBDE | 5 | + | **<0.26 - 5.5** | ng/g w.w. | Leslie et al. 2011 |
| ns | ns | Field | Muscle | PBDEs | 23 | + | **120 - 8500** | ng/g w.w. | Yu et al. 2011 |
| ns | ns | Field | Liver | Cd | 7 | + | **<LOD - 6.23** | µg/g w.w. | Zaccaroni et al. 2011 |
| ns | ns | Field | Liver | Hg | | + | **<LOD - 25.6** | µg/g w.w. | |
| ns | ns | Field | Liver | Pb | | + | **<LOD - 0.90** | µg/g w.w. | |
| ns | ns | Field | Liver | Zn | | + | **23.5 - 224.6** | µg/g w.w. | |
| ns | ns | Field | Liver | Cu | | + | **1.66 - 42.9** | µg/g w.w. | |
| ns | ns | Field | Kidney | Cd | | + | **<LOD - 13.87** | µg/g w.w. | |
| ns | ns | Field | Kidney | Hg | | + | **<LOD - 4.24** | µg/g w.w. | |
| ns | ns | Field | Kidney | Pb | | + | **<LOD - 0.22** | µg/g w.w. | |
| ns | ns | Field | Kidney | Zn | | + | **17.87 - 565** | µg/g w.w. | |
| ns | ns | Field | Kidney | Cu | | + | **1.68 - 15.7** | µg/g w.w. | |
| ns | ns | Field | Bone | Cd | | - | **<LOD** | µg/g w.w. | |
| ns | ns | Field | Bone | Hg | | + | **<LOD - 0.74** | µg/g w.w. | |
| ns | ns | Field | Bone | Pb | | + | **<LOD - 5.08** | µg/g w.w. | |
| ns | ns | Field | Bone | Zn | | + | **52.6 - 514** | µg/g w.w. | |
| ns | ns | Field | Bone | Cu | | + | **1 - 9.44** | µg/g w.w. | |
| Adult | ns | Field | Liver | Pb | 2 | + | **4.3 - 8.3** | ppm | Nam et al. 2011 |
| Adult | ns | Field | Kidney | Pb | | + | **9.4 - 45.0** | ppm | |
| Adult | ns | Field | Bone | Pb | | + | **9.0 - 11.6** | ppm | |

**Table A3: Results of articles on body condition. The abbreviation *ns* means "not specified". The symbol "+" indicates an effect on the body condition, while the symbol "-" indicates no effect.**

| Life stage | Sex | Study type | Biological material | Contaminant | Human exploited environments | Methodological approach | Sample size | Results | | Reference, year |
|---|---|---|---|---|---|---|---|---|---|---|
| Nestling | ns | Field | Animal | | Urban | Body condition | 143 | + | | Wemer et al. 2021 |
| Adult | ns | Field | Animal | | Agricultural | Body condition | 448 | - | | Sumasgutner et al. 2019 |
| Nestling | ns | Field | Animal | | Urban | Body condition | 154 | - | | Sumasgutner et al. 2018 |
| Nestling | ns | Field | Animal | Bromadiolone | | Body condition | 112 | + | | Martínez-Padilla et al. 2017 |
| Nestling | ns | Field | Animal | | Agricultural | Body condition | 248 | + | **160-190** | Costantini et al. 2014 |

**Table A4: Results of article on poisoning events. The abbreviation *ns* means "not specified". The symbol "+" indicates poisoning occurrences.**

| Life stage | Sex | Study type | Biological material | Contaminant | Sample size | Results (min-max; N) | | UM | Reference, year |
|---|---|---|---|---|---|---|---|---|---|
| ns | ns | Hystorical database | Animal | ns | 9 | + | **0 - 20** | % | Gil-Sánchez et al. 2021 |
| | | Field | | | 3 | + | **0 - 0.5** | | |
| ns | ns | Field | Animal | Carbofuran | 17 | + | **3** | n | Ruiz-Suárez et al. 2015 |
| | | | | Bromadiolone, Brodifacoum and Difenacoum | | + | **4** | | |
| | | | | Aldicarb | | + | **4** | | |
| | | | | Oxamyl, Methomyl, Chlorpyrifos, Diazinon, Fenthion and Propoxur | | + | **3** | | |
| | | | | Fipronil, Carboxin and Fenazaquin | | + | **1** | | |
| ns | ns | Field | Liver | Bromadiolone, Brodifacoum, Difenacoum | 22 | + | | | Hughes et al. 2013 |

**Table A5: Results of articles on parasitology. The abbreviation *ns* means "not specified". The symbol "-" indicates no presence of parasites.**

| Life stage | Sex | Study type | Biological material | Contaminant | Human exploited environments | Methodological approach | Sample size | Results | | Reference, year |
|---|---|---|---|---|---|---|---|---|---|---|
| Nestling | ns | Field | Animal | | Urban | Ectoparasite infection | 143 | - | | Wemer et al. 2021 |
| Adult | ns | Field | Blood | | Agricultural | Blood parasite infection | 448 | - | | Sumasgutner et al. 2019 |
| Nestling | ns | Field | Animal | | Urban | Ectoparasite infection | 154 | - | | Sumasgutner et al. 2018 |

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**Supplementary material**

# A blood-based multi-biomarker approach reveals different physiological responses of common kestrels to contrasting environments

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**Table S1.** Mean ± SD and range values of biomarkers with relative sample size (N) of common kestrel nestlings sampled from three study areas (natural = NAT, rural = RUR, urban = URB).

| **Biomarker** | **NAT** | | | **RUR** | | | **URB** | | |
|---|---|---|---|---|---|---|---|---|---|
| | **Mean±SD** | **min-max** | **N** | **Mean±SD** | **min-max** | **N** | **Mean±SD** | **min-max** | **N** |
| **GSH** µM | 3162.45 ± 2270.26 | 101.86 – 6786.80 | 16 | 4997.02 ± 5136.33 | 109.27 – 12657.50 | 18 | 2175.22 ± 5084.33 | 51.09 – 12553.12 | 6 |
| **GSSG** µM | 15.93 ± 15.18 | 0.00 – 50.40 | 16 | 19.70 ± 20.61 | 0.00 – 61.24 | 18 | 24.45 ± 24.13 | 0.00 – 66.53 | 6 |
| **GSH:GSSG** | 175.14 ± 152.56 | 6.64 – 429.03 | 12 | 470.13 ± 838.80 | 3.77 – 3086.65 | 14 | 193.07 ± 420. 48 | 1.87 – 945.22 | 5 |
| **TAS** mM Trolox | 8.97 ± 8.77 | 0 – 22.92 | 20 | 7.27 ± 6.38 | 0 – 22.04 | 21 | 13.89 ± 11.72 | 0 – 26.53 | 7 |
| **Respiratory Burst** O.D. 630 nm | 0.32 ± 0.63 | 0.04 – 2.53 | 18 | 0.20 ± 0.44 | 0.04 – 2.09 | 22 | 0.49 ± 0.66 | 0.16 – 1.68 | 5 |
| **Differential WBCs Count** | | | 28 | | | 27 | | | 5 |
| *Heterophils* N/200 leukocytes | 65.71 ± 25.45 | 20 – 156 | 28 | 69.70 ± 25.30 | 28 – 119 | 27 | 60.20 ± 22.43 | 34 – 90 | 5 |
| *Eosinophils* N/200 leukocytes | 6.68 ± 5.58 | 1 – 21 | 28 | 13 ± 14.32 | 0 – 61 | 27 | 13.80 ± 6.38 | 3 – 18 | 5 |
| *Basophils N/200 leukocytes* | 0.00 ± 0.00 | 0.00 – 0.00 | 28 | 0.00 ± 0.00 | 0.00 – 0.00 | 27 | 0.00 ± 0.00 | 0.00 – 0.00 | 5 |
| *Lymphocytes* N/200 leukocytes | 121.68 ± 25.87 | 42 – 166 | 28 | 111.19 ± 27.15 | 70 – 164 | 27 | 115.40 ± 18.90 | 97 – 140 | 5 |
| *Monocytes* N/200 leukocytes | 3.44 ± 4.18 | 0 – 16 | 27 | 6.11 ± 5.19 | 0 – 21 | 27 | 10.60 ± 2.30 | 8 – 13 | 5 |
| *Heterophils* % | 31.72 ± 13.91 | 0.00 – 78.00 | 28 | 33.61 ± 14.05 | 0.00 – 59.50 | 27 | 25.08 ± 15.86 | 0.00 – 45.00 | 5 |
| *Eosinophils* % | 3.22 ± 2.81 | 0.00 – 10.50 | 28 | 6.27 ± 7.13 | 0.00 – 30.50 | 27 | 5.75 ± 4.01 | 0.00 – 9.00 | 5 |

| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| *Basophils*<br>*%* | 0.00 ± 0.00 | 0.00 – 0.00 | 28 | 0.00 ± 0.00 | 0.00 – 0.00 | 27 | 0.00 ± 0.00 | 0.00 – 0.00 | 5 |
| *Lymphocytes*<br>% | 58.74 ± 17.00 | 0.00 – 83.00 | 28 | 53.61 ± 16.97 | 0.00 – 82.00 | 27 | 48.08 ± 25.03 | 0.00 – 70.00 | 5 |
| *Monocytes*<br>% | 1.66 ± 2.08 | 0.00 – 8.00 | 27 | 2.95 ± 2.61 | 0.00 – 10.50 | 27 | 4.42 ± 2.40 | 0.00 – 6.50 | 5 |
| **H/L ratio** | 0.64 ± 0.64 | 0.12 – 3.71 | 28 | 0.72 ± 0.41 | 0.17 – 1.59 | 27 | 0.56 ± 0.28 | 0.24 – 0.93 | 5 |
| **Complement system activity**<br>Haemolysis % | 32.72 ± 25.96 | 5.37 – 98.61 | 20 | 31.89 ± 23.86 | 6.12 – 75.65 | 20 | 17.69 ± 8.77 | 6.20 – 27.74 | 7 |
| **Bactericidal ability**<br>% | 28.00 ± 13.14 | 0 – 17.52 | 13 | 35.00 ± 20.24 | 0.57 – 70.29 | 16 | 23.00 ± 4.12 | 1.56 – 61.14 | 4 |
| **Comet assay**<br>DNA tail % | 3.35 ± 1.83 | 0.90 – 7.09 | 26 | 4.70 ± 1.93 | 1.38 – 9.81 | 28 | 3.33 ± 0.68 | 2.17 – 4.41 | 8 |
| **ENAs assay**<br>Σ N abnormalities | 133.67 ± 58.88 | 41 – 238 | 30 | 162.11 ± 48.55 | 77 – 251 | 28 | 131.67 ± 47.94 | 66 – 197 | 5 |
| *Bud*<br>N/2000 cells | 72.33 ± 34.71 | 26 – 146 | 30 | 88.11 ± 36.67 | 33 – 174 | 28 | 81.17 ± 23.96 | 42 – 117 | 5 |
| *Segmented*<br>N/2000 cells | 21.63 ± 16.72 | 2 – 67 | 30 | 26.54 ± 16.32 | 7 – 67 | 28 | 13.67 ± 10.67 | 2 – 30 | 5 |
| *Notched*<br>N/2000 cells | 22.87 ± 11.34 | 4 – 39 | 30 | 28.43 ± 13.02 | 4 – 63 | 28 | 21.50 ± 13.31 | 9 – 42 | 5 |
| *Kidney*<br>N/2000 cells | 16.60 ± 8.01 | 1 – 27 | 30 | 18.43 ± 7.03 | 3 – 32 | 28 | 15.33 ± 5.54 | 9 – 22 | 5 |
| *MN*<br>N/2000 cells | 0.23 ± 0.50 | 0 – 2 | 30 | 0.61 ± 0.88 | 0 – 3 | 28 | 0.00 ± 0.00 | 0 – 0 | 5 |
| **BChE activity**<br>µmol/min/mL | 0.24 ± 0.11 | 0.00 – 0.37 | 17 | 0.33 ± 0.07 | 0.23 – 0.45 | 19 | 0.20 ± 0.06 | 0.10 – 0.25 | 5 |
| **CaE activity**<br>nmol/min/mL | 89.50 ± 23.77 | 47.95 – 126.19 | 16 | 100.36 ± 15.92 | 79.19 – 135.36 | 18 | 92.10 ± 16.26 | 65.45 – 109.56 | 5 |

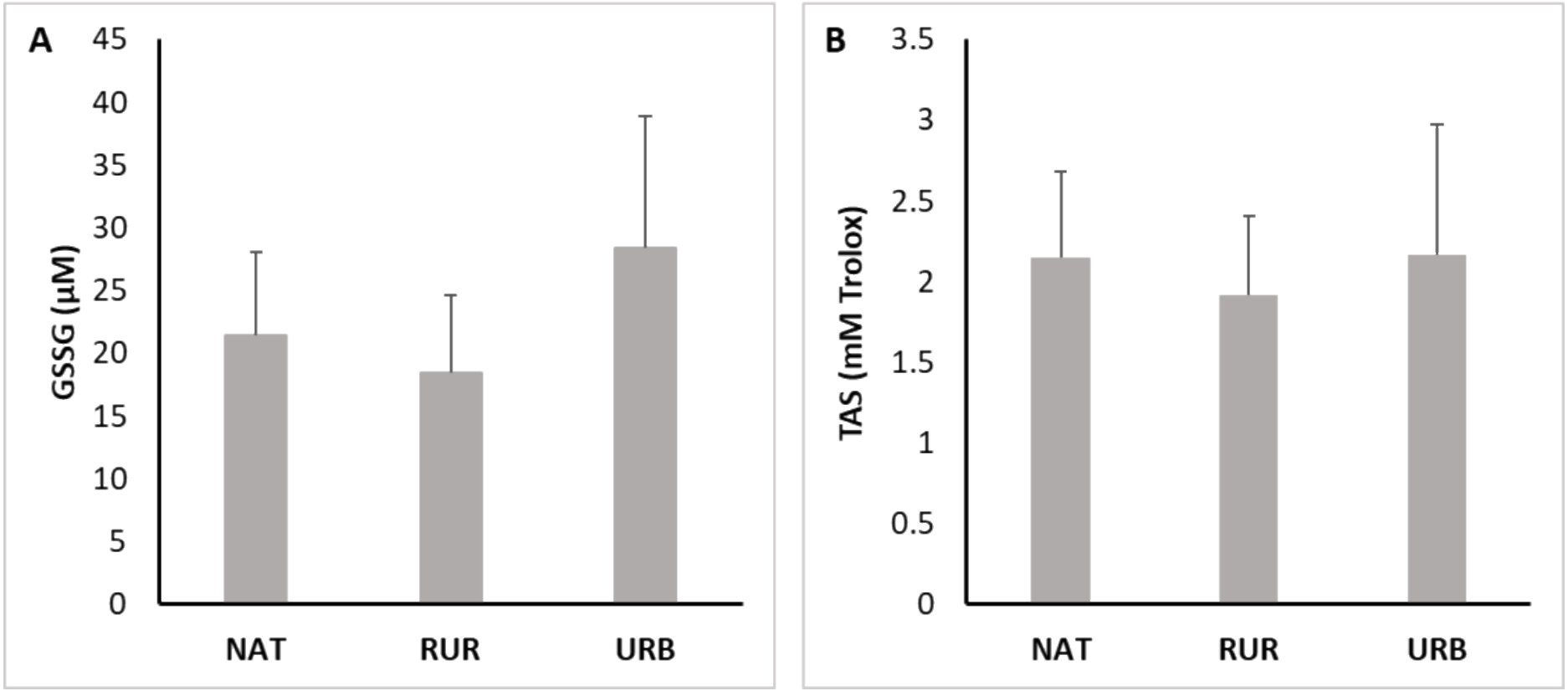


**Figure S1.** GSSG (A) and TAS assay (B) results of common kestrel nestlings sampled in NAT= natural, RUR= rural, and URB= urban areas. Results are presented as emmean ± SE.

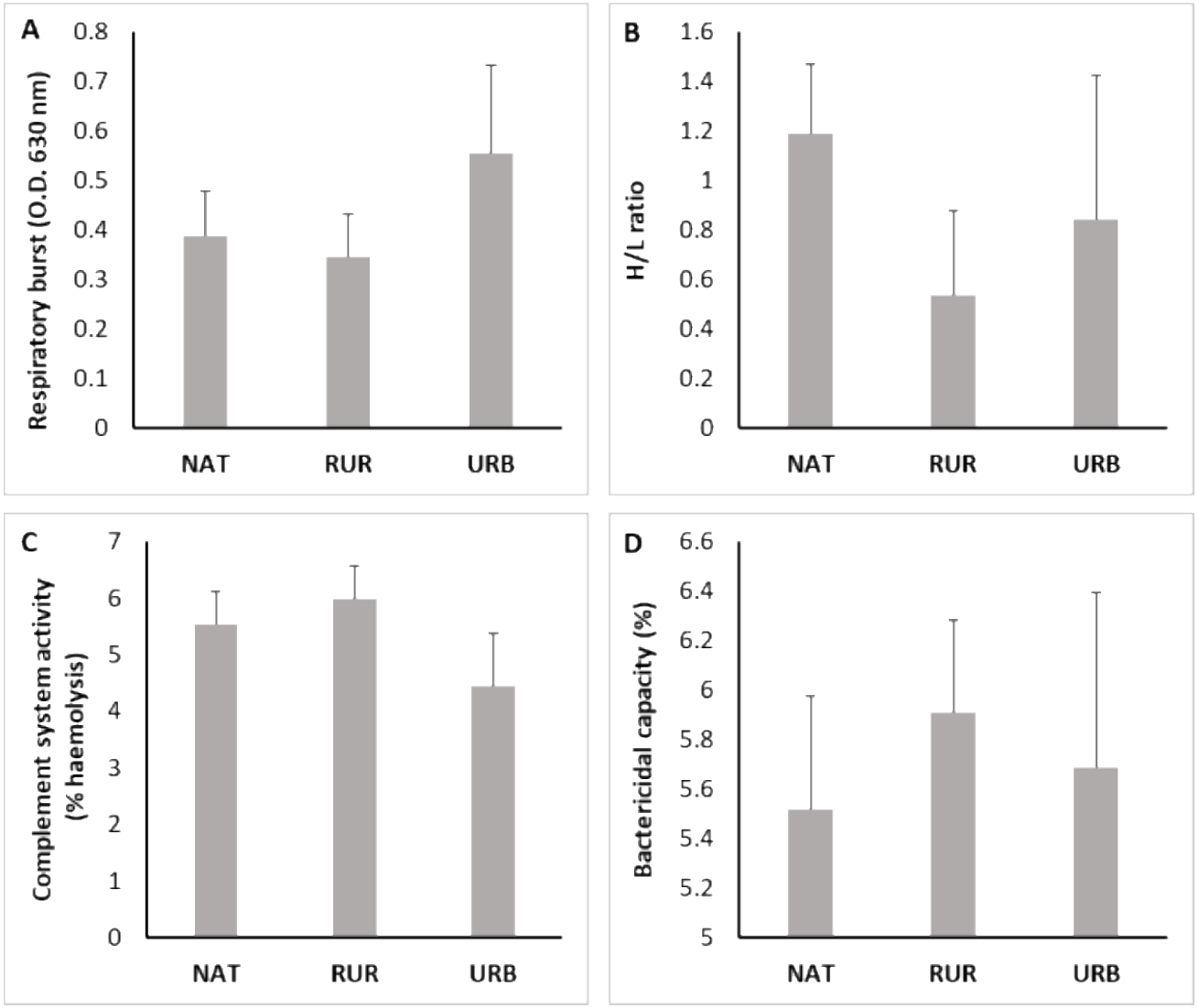


**Figure S2.** Respiratory burst (A), H/L ratio (B), Complement system activity (C) and Bactericidal capacity (D) results of common kestrel nestlings sampled in NAT= natural, RUR= rural, and URB= urban areas. Results are presented as emmean ± SE.

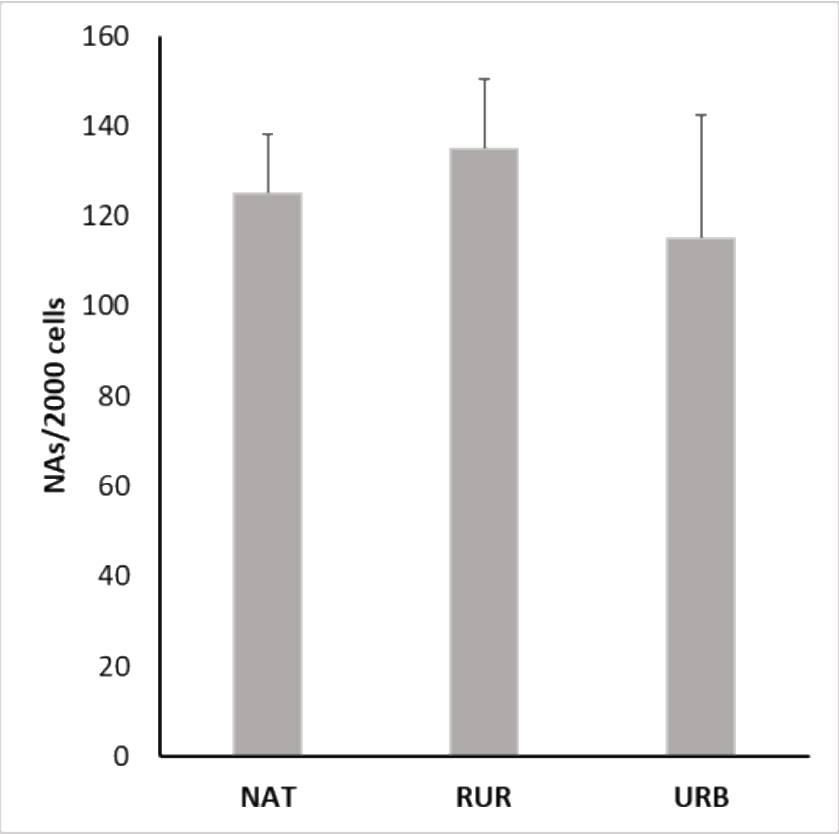


**Figure S3.** Total ENAs results of common kestrel nestlings sampled in NAT= natural, RUR= rural, and URB= urban areas. Results are presented as emmean ± SE.

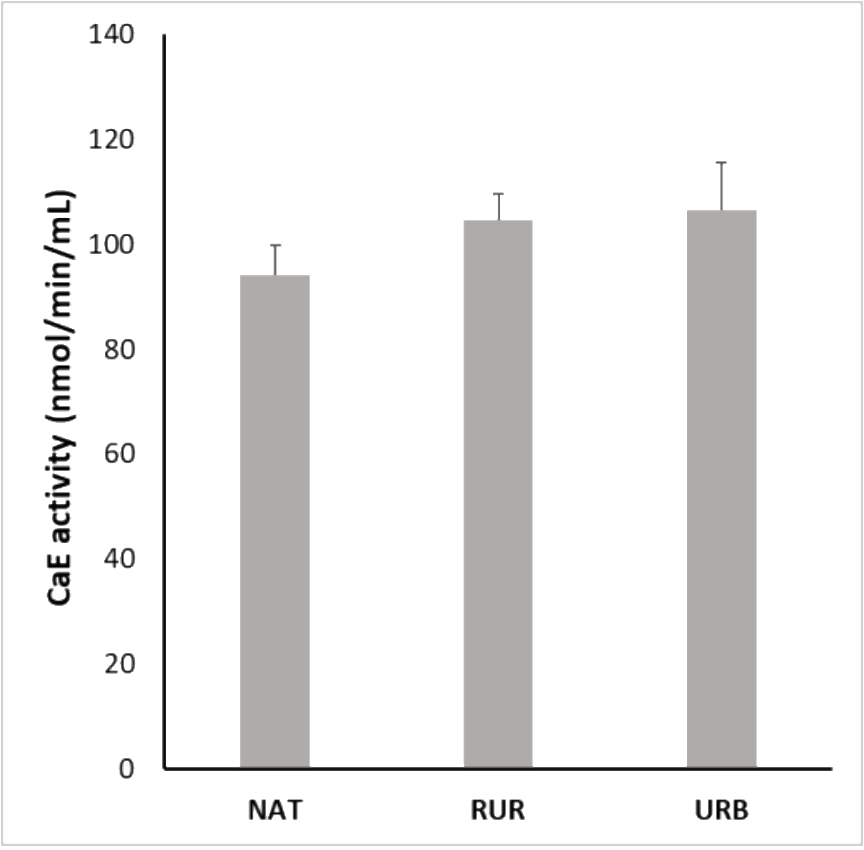


**Figure S4.** Carboxylesterase activity results of common kestrel nestlings sampled in NAT= natural, RUR= rural, and URB= urban areas. Results are presented as emmean ± SE.

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**ACKNOWLEDGMENTS**

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