



A multi-biomarker approach to evaluate the ecotoxicological status of great tit (*Parus major*) populations from areas with different anthropogenic impacts

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ABSTRACT

Multiple anthropogenic pressures can lead to physiological alterations or damages to birds, potentially resulting in cascading effects on individual's fitness and population dynamics. Cavity-nesting birds, such as the great tit (*Parus major*), provide an excellent model for investigating the effects of environmental stressors on avian species. Their territorial behaviour and the accessibility of their nests make great tits particularly suitable for investigating the environmental conditions of their nesting locations. This study aimed to evaluate the ecotoxicological status of great tit populations inhabiting areas with different anthropogenic impacts, using a multi-biomarker approach. During the breeding seasons of 2021 and 2022, blood and excreta samples were collected from 198 nestlings belonging to 44 nests across four different areas of the Veneto region (northern Italy), representing wood (14 nests), urban (9 nests) and agricultural (9 and 12 nests) environments. Immunotoxicity, oxidative status, genotoxicity and neurotoxicity responses were evaluated on blood, while porphyrins levels on excreta. Results revealed significant physiological alterations in nestlings from urban and agricultural areas (oxidative status modifications, immune response impairments and genotoxic effects), likely associated with environmental stressors typical of anthropized ecosystems. The wood area presented elevated protoporphyrin levels, suggesting unexpected exposure to diffuse airborne particulate matter from nearby cities. The IBR index revealed that populations inhabiting urban and agricultural environments suffered the highest physiological stress. This multi-biomarker approach has proved to be a valid tool for assessing the ecotoxicological status of the great tit populations exposed to different stressors, allowing to be proposed for the conservation of wild bird species.

1. Introduction

The escalation of anthropogenic activities, including urbanization, industrialization, and intensive agriculture, drives habitat loss and fragmentation (Liu et al., 2016; Williams et al., 2021), triggers the release of massive quantities of pollutants into the environment (Tudi et al., 2021; Ding et al., 2023), and exacerbates global climate change (Dubey et al., 2023; Tscharnkte & Batáry, 2023). These interconnected pressures induce both temporary discomforts and permanent

physiological damages in biota, including avian species (Reynolds et al., 2019; Richard et al., 2021; Douglas et al., 2023; Rigal et al., 2023). Specifically, reductions in food availability and quality (Gibbons et al., 2006), habitat degradation (Dunn & Møller, 2019; Dri et al., 2021), and direct human disturbance significantly impair individual fitness and population dynamics.

Birds are widely recognized as excellent bioindicators of environmental health (Hao et al., 2021). Their apical position in the food chain makes them susceptible to bioaccumulation and biomagnification

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processes (Francis, 2017; Baesse et al., 2019) and their wide distribution exposes them to multiple stressors present in the different environmental compartments and matrices (Bårdsen et al., 2018). Moreover, given their relative ease of field study (Smits & Fernie, 2013), birds facilitate the application of non-destructive techniques for collecting samples such as blood, feathers, excreta, and unhatched eggs (García-Fernández, 2014). This non-destructive sampling strategy not only allows for an effective, continuous monitoring of avian health without compromising animal health status, but also enables the implementation of long-term monitoring programs (Casini et al., 2018; Bjedov et al., 2025).

Historically, wildlife biomonitoring in anthropogenic environments has predominantly relied on restricted biological responses (e.g., focusing on a single physiological system) (Geens et al., 2010; Koivula et al., 2011; Ouyang et al., 2013; Pollack et al., 2017) or on macro-fitness indicators such as reproductive success (Sens et al., 2003; de Satgé et al., 2019; Wawrzyniak et al., 2020). This traditional framework oversimplifies the complexity of ecotoxicological responses, failing to capture sub-lethal, non-linear effects and the cumulative impact of multiple co-occurring pressures. To date, field studies evaluating and integrating diverse physiological responses remain critically limited.

In this context, to bridge this gap and to achieve a more comprehensive overview of the population health status, biomarkers can represent a sensitive and useful tool to measure biological effects and assess environmental conditions across varying habitats and disturbance gradients (García-Fernández, 2014). Biomarkers offer valuable insights into the potential additive or synergistic interactions of multiple anthropogenic stressors (Martínez-Haro et al., 2015; Milinkovitch et al., 2019). Concurrently, multi-biomarker responses facilitate the assessment of both short-term acute stress responses and long-term cumulative damage or pollutant accumulation (Lionetto et al., 2019). Consequently, biomarker-based monitoring programs can seamlessly integrated into modern ecotoxicology and avian conservation strategies.

The great tit (*Parus major* Linnaeus, 1758) is a passerine bird species widely distributed across diverse habitats (Cusimano et al., 2025), from forested areas to urban green spaces (Bailly et al., 2016). Moreover, great tits breed in artificial nest-boxes (Chmielewski et al., 2013), which facilitates reproduction monitoring and sample collection. This makes them an ideal model to investigate the localized effects of anthropogenic pressures on specific wild populations. However, despite the widespread use of the great tit as a model species, most monitoring studies still focus on a limited number of endpoints (Koim-Puchowska et al., 2020; Groffen et al., 2024; Santos et al., 2025), rather than employing a multi-biomarker approach that would provide a more comprehensive understanding of their health status.

In this study, we assessed the ecotoxicological status of great tit populations inhabiting areas subjected to different anthropogenic impacts. In particular, a multi-biomarker approach was applied to assess sub-lethal physiological alterations in great tit nestlings inhabiting areas subjected to contrasting anthropogenic pressures, ranging from a wooded environment to urban and agricultural settings. Specifically, this study aimed to evaluate a suite of molecular, biochemical and cellular level toxicological responses in great tit nestlings, including immunotoxicity (complement system activity, respiratory burst and differential White Blood Cells (WBCs) count), oxidative status (Total Antioxidant Status (TAS) assay), genotoxicity (comet assay and Erythrocytes Nuclear Abnormalities (ENA) assays), neurotoxicity effects (butyrylcholinesterase (BChE) and carboxylesterase (CaE) activities) and porphyrin levels.

We focused on oxidative status (Total Antioxidant Status assay), because the test allows to measure plasmatic antioxidants capable of inhibiting a redox reaction induced by free radicals (Bourgeon et al., 2012; Cohen et al., 2007). The respiratory burst is a process that plays an important role in the innate immune response to pathogen infection and it also may induce increasing levels of pro-oxidant that can cause

oxidative stress to the animal. Since the immune system of the great tit has been shown to be affected by various environmental stressors, we evaluated the complement system activity, a fundamental component of the innate immune system. It plays a key role in normal physiology of a healthy organism (e.g., maintenance of cell survival), triggering inflammatory responses, and in the defence against pathogens (Merle et al., 2015). The differential white blood cells count (WBCs) was used to evaluate immune function and physiological stress. Specifically, the ratio between heterophiles and lymphocytes (H/L) is an index commonly used as indicator of long-term stress and health changes (Davis et al., 2008; Parejo and Silva, 2009). We evaluated the alkaline comet assay because it is a rapid and highly sensitive method to quantify DNA alterations in nucleated erythrocytes as an early biomarker of environmental genotoxicity (Cortés-Gutiérrez et al., 2026). Moreover, this method is also been validated for its employment as a monitor of avian species in contaminated lands (Pastor et al., 2004). To identify possible chromosomal aberrations (micronuclei and other nuclear abnormalities) in the erythrocyte nuclei generated during mitosis by the chromosome breakage or failure cytokinesis (Frixione and Rodríguez-Estrella, 2020), we assessed ENA assay. To provide early responses to pesticide exposure (Anglister et al., 2023), butyrylcholinesterase (BChE) and carboxylesterase (CaE) enzymes were performed. Furthermore, porphyrins were evaluated as their accumulation can indicate exposure to toxic agents (Casini et al., 2003). This integrative approach aims to provide a comprehensive understanding of how complex mixtures of environmental stressors affect great tits health, with potential implications for conservation and environmental monitoring.

2. Material and methods

2.1. Study areas

The study was conducted on great tits populations breeding in different areas of Padua province (Veneto Region, North-East Italy), from the middle of March to the end of May, for two consecutive years (2021–2022). Based on the varying types of anthropogenic influence, the areas were classified as wooded, urban, and agricultural environments. Specifically, the Regional Natural Park of the Euganean Hills was selected as the “wood area”. This area is characterized by hills of volcanic origin and dominated by deciduous vegetation (601 m a.s.l.), located in the southwestern part of the Province of Padua, approximately 15 km from the city centre. The “urban area” included four sites located in the centre of Padua city and its proximity (Vallisneri, Esapolis and Padua's botanical garden, S. Antonio Village-Noventa Padovana). According to the European Environment Agency (EEA, 2025), Padua is considered one of the most air-polluted cities in Europe, based on the population-weighted concentrations of particulate matter (PM_{2.5}), nitrogen dioxide (NO₂), and ozone (O₃), which account for pollutant levels adjusted to population distribution. Cà di Mezzo Oasis and Vaccarino were used as agricultural areas, distinguished in agricultural 1 (hereinafter “agri 1”) and agricultural 2 (hereinafter “agri 2”), respectively (Fig. S1). Cà di Mezzo Oasis (agri 1) is an artificial phytodepuration site that collects the wastewater of a canal that drains water from upstream farmland and is surrounded by intensive cultivation of wheat and corn. This area is also near the Bacchiglione and Brenta rivers, both of which have been subject to contamination of agricultural and industrial origin over the years (Masiol et al., 2018; Bonato et al., 2020; Mirauda et al., 2021). Vaccarino site (agri 2), 15 km away from Padua's city centre, is bordered by wheat crops and is relatively close to both a busy road and the Brenta River. Further details regarding the characteristics of the study areas are reported in the Supplementary Material.

2.2. Field routine and sample collection

Field monitoring was performed using artificial nest boxes. To

minimize structural and microclimatic confounding effects, all nest boxes were identical in material and dimensions and were installed at a standardized mean height of 3 m above the ground with a Southeast orientation. Nest boxes were checked every 5 days to monitor reproduction and to annotate lay date, clutch size, hatching dates and brood sizes at hatching and fledging. All nestlings were weighted (to the nearest 0.01 g) with a digital balance at day 15 of life (hatching day = day 1).

For two years (2021 and 2022) we sampled blood ($N = 198$) and excreta ($N = 143$) from great tit nestlings, in a total of 44 nests (19 in 2021 and 25 in 2022). Specifically, in both years, we sampled a total number of 66 nestlings from the wood area (14 nests), 40 from the urban area (9 nests), 42 from the agri 1 site (9 nests) and 50 from the agri 2 area (12 nests). For nestlings sampling, nests were randomly selected and the sampling was performed between 8 a.m. and 4 p.m. Blood sampling was performed in 15 days nestlings through venipuncture in the brachial vein. Samples were collected with a heparinized capillary tube and a drop of fresh blood was used to make blood smears (two per nestling). The remaining blood was transferred in a 500 μL vacutainer (MICROMED®, Italy) containing lithium-heparin as an anticoagulant and processed. Blood samples were divided into different aliquots: an aliquot was frozen, a second aliquot was mixed with a cryoconservation medium (RPMI:DMSO, 80:20) for the comet assay and the remaining whole blood was centrifuged at 6000 g (Multispin 12, Argo LAB, Carpi, Italy) for 10 min to separate plasma from red cells. All the blood and plasma samples were stored at -80°C until analysis. Excreta were collected in tubes, and stored at -20°C .

2.3. Biomarker analysis

Immunotoxicity, oxidative status, genotoxicity and neurotoxicity effects were evaluated in the blood of the nestlings, while porphyrins levels were measured in excreta.

2.3.1. Immunotoxicity

2.3.1.1. Complement system activity. Plasma complement activity was determined by the spectrophotometric method of sheep red blood cells (SRBCs) hemolysis following the protocol of Merchant and Britton (2006). The whole blood of healthy Merino sheep (*Ovis aries*) was centrifuged at 3000 $\times$ g to obtain fresh SRBCs. The SRBCs were washed with phosphate-buffered saline (PBS, pH 7.4) and then diluted to 2% (v/v) in PBS. Plasma samples were incubated with an equal volume of 2% SRBCs (v/v) for 30 min at 37°C . Afterwards, samples were centrifuged (2500 $\times$ g for 5 min) and the supernatant was used in a microplate reader at 540 nm (Multiskan Skyhigh Thermo Scientific). A positive control was prepared by adding Triton X-100 to 1% SRBCs suspension and homogenized until complete hemolysis; then, it was centrifuged and the optical density (O.D.) measured (considered 100% hemolysis). The results were expressed as mean % hemolysis [(O.D. sample/O.D. positive control) $\times$ 100].

2.3.1.2. Respiratory burst. The respiratory burst activity was evaluated as the production of intracellular oxyradicals by NADPH oxidase and measured using the Nitroblue Tetrazolium (NBT) assay, adapted from Caliani et al. (2019). Whole blood aliquots (20 μL) from each sample were transferred into duplicate wells of a 96-well plate and incubated at 40°C for 2 h to allow blood cell adhesion to the well surface. Non-adherent cells were subsequently removed by washing the wells three 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) were added to each well to stimulate NADPH oxidase system of the cells and the plate was incubated at room temperature for 1 h. After incubation, the supernatant was discarded and the plate was fixed with 100% methanol for 10 min. The plate was then washed with 70%

methanol and air-dried. 120 μL of KOH and 140 μL of DMSO were added to each well to dissolve the crystals of formazan blue deriving from the reduction of NBT by the oxyradicals. Absorbance was measured at 630 nm using a multi-plate reader (Multiskan Skyhigh Thermo Scientific) using KOH/DMSO as blank. The respiratory burst activity was expressed as a reduction of NBT (optical density at 630 nm).

2.3.1.3. Differential white blood cells (WBCs) count and H/L ratio. Air-dried blood smears were stained with Diff-Quick stain (Mgg Quick Stain, Bio-Optica), air-dried and analysed using an optical immersion microscope (Olympus BX41) at 100 $\times$ magnification for differential WBCs count and ENA assay.

Two hundred leukocytes were counted manually (two replicates per sample) with an optic microscope (Olympus BX41) at 100 $\times$ magnification and the cells were classified as heterophils, eosinophils, basophils, lymphocytes and monocytes according to the cellular morphology for birds described by Campbell (1995). Results were expressed as the number of each leukocyte category per 200 leukocytes counted.

The number of heterophils and lymphocytes counted in each animal was also used to calculate the H/L ratio, an index of physiological stress (Davis et al., 2008).

2.3.2. Oxidative status

2.3.2.1. TAS assay. The TAS was evaluated using a commercial kit (Antioxidant Assay Kit, Sigma, St. Louis, MO) by the spectrophotometric method of Miller et al. (1993), 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 (0.015, 0.045, 0.105, 0.21, 0.42 mM). 10 μL of Trolox concentration aliquots and plasma samples (diluted 1:100 in assay buffer) were added to a 96-well plate in duplicate. 20 μL myoglobin and 150 μL of the chromogen ABTS (2,2'-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) were added to each well and the plate was incubated at room temperature for 4.5 min. Absorbance at 405 nm was measured using a multi-plate reader (Multiskan Skyhigh Thermo Scientific). The TAS was expressed as mM of Trolox by linear regression of the standard curve.

2.3.3. Genotoxicity

2.3.3.1. 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 . The slides were then placed on a horizontal electrophoresis tray previously filled with freshly prepared cold alkaline buffer (0.3 M NaOH, 1 mM EDTA, pH 13) and left for 15 min to allow DNA unwinding. Electrophoresis was performed at 16 V (0.6 V/cm) and 250 mA for 10 min. Slides were then neutralized with 0.4 M Tris (pH 7.5) and stained with SYBR®Safe 1:10.000 in TE buffer (10 mM Tris-HCl pH 7.5 and 500 mM EDTA pH 7.5). 100 cells per sample were examined under the epifluorescence microscope (Olympus BX41) at 40 $\times$ magnification. The amount of DNA damage was evaluated as the DNA tail % using an image analyzer (6.0 Software, Kinetic Imaging Ltd.).

2.3.3.2. ENA assay. Quantification of ENAs was conducted in the same slides used for the differential leukocyte count according to the method of Frixione and Rodríguez-Estrella (2020). A total of 2000 mature erythrocytes per sample were analysed and quantified at 100 $\times$ magnification using an optical immersion microscope (Olympus BX41). The nuclear alterations were classified as follows: bud, segmented, notched, kidney and micronucleus. Results were expressed as the number of each

anomaly per 2000 erythrocytes and as the total number of abnormalities per 2000 cells.

2.3.4. Neurotoxicity

2.3.4.1. BChE activity. Butyrylcholinesterase activity was evaluated in plasma samples using the spectrophotometric test of Fossi et al. (1992), modified. The reaction assay mixture contained: 25 mM TRIS-HCl buffer (pH 7.6), 1 mM CaCl₂, DTNB (5, 5'dithiobis 1-2-nitrobenzoic acid), 10 μ L BTCl and 2 μ L of plasma. The activity was measured at 410 nm (40 °C) with a micro-plate reader (Multiskan Skyhigh Thermo Scientific) for 5 min and expressed as μ mol min⁻¹ mL⁻¹ plasma (ϵ = 13,6 mmol/L).

2.3.4.2. CaE activity. Carboxylesterase activity was evaluated with the spectrophotometric method of Solé et al. (2022) with minor modifications. To a 96-well 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 μ L of plasma. Samples and blank were run in double in a micro-plate reader (Multiskan Skyhigh Thermo Scientific) measuring the absorbance at 405 nm (40 °C) for 5 min. The activity was expressed as nmol min⁻¹ mL⁻¹ plasma (ϵ = 18000 M⁻¹ cm⁻¹).

2.3.5. Porphyrins

The excreta samples were freeze-dried and then transformed into powder through a pestling process. Extraction of acid porphyrins from excreta was carried out using the method from Lockwood et al. (1985). For each sample, 50 mg of excreta were added to 1 mL of HCl 5N, 3 mL of diethyl ether and 3 mL of distilled water. Finally, the samples were centrifuged at 2700 x g for 5 min, obtaining three different layers; a supernatant of diethyl ether where carotenoid derivatives are located, a central layer of insoluble material and a watery sedimented portion where porphyrins are contained. The porphyrins were carried out by Grandchamp et al. (1980) method, which determine the concentration of copro-, uro-, 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). Concentrations were expressed as pmol g⁻¹ dry weight of excreta.

2.4. 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 characterized by different anthropogenic impacts on the biological responses of great tit nestlings, a series of univariate linear or generalised linear mixed models were applied (one model for each biomarker). In particular, the single biomarker was always included as a dependent variable, while the “area” was included as a fixed factor (4 levels: wood, urban, agri 1 and agri 2). “Body mass” and Julian “sampling date” were included as covariates to correct for any possible effect of nestlings' condition and breeding time, respectively. Moreover, “year” (2 levels: 2021/2022) was included as fixed effect to correct for the possible effect of the different breeding seasons. Finally, “nest identity” was included as random effect to correct for the non-independence among chicks from the same clutch. All covariates (i.e. body mass and sampling date) were Z-transformed to have a mean of 0 and an SD of 1 (function ‘scale()’).

First, regarding oxidative status biomarker, we performed a linear mixed model (LMM, function “lmer()”, package “lme4”) with “TAS” as dependent variables, while about porphyrins, a series of LMMs was applied to “coproporphyrins”, “uroporphyrins”, “protoporphyrins” and “total porphyrins”. To reach the normality of residuals, a log-transformation was applied for coproporphyrins, while a square-root transformation was applied for uroporphyrins and protoporphyrins.

Second, regarding immunotoxicity 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 “lme4”) was instead applied for count variables such as “heterophils”, “eosinophils”, “lymphocytes” and “monocytes”. In this case, a Poisson error distribution was considered, and an observation-level random effect was included in “eosinophils”, “lymphocytes” and “monocytes” models to control for overdispersion (Harrison, 2014; Pearson's Chi-squared test: $p < 0.05$). Third, regarding genotoxicity biomarkers, we fitted a LMM for the “comet assay”. In this case, a square-root transformation was applied to achieve normality of the residuals. GLMMs with a Poisson error distribution were 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 Pearson's Chi-squared test ($p < 0.05$). Finally, for neurotoxicity biomarkers, we performed two LMMs with “BChE” and “CaE” as dependent variables. In this case, to achieve residual normality, a log-transformation was applied for neurotoxicity biomarkers (BChE, CaE) only. Mean, standard deviation, min e max of all biomarkers, are reported in the supplementary materials (Table S1). Outcomes from univariate linear or generalised linear mixed models are showed in supplementary materials (Table S2–S6). Significance was determined at the $\alpha = 0.05$ level.

To provide an integrative perspective on the overall biological response of great tit nestlings across study areas, and to summarise multiple physiological endpoints into a single score that reflects the overall ecotoxicological status of each population (Beliaeff and Burgeot, 2002; Sanchez et al., 2013), an Integrated Biomarker Response (IBR) index was calculated following the method of Beliaeff and Burgeot (2002). The IBR was computed using individual-level data for all biomarkers measured in blood and excreta collected from great tit nestlings. The IBR was calculated using the following steps. First, for each biomarker, individual values were used to compute the mean per study area. Biomarkers for which low values indicate biological stress (BChE, respiratory burst, complement system) were multiplied by -1 , so that for all biomarkers higher values consistently reflect greater physiological alteration (Devin et al., 2014). This procedure ensures that the IBR score reflects the overall degree of biological alteration in a biologically meaningful and directionally consistent manner, where higher IBR values indicate greater ecotoxicological impairment. The means were then standardized using a z-score transformation, where the mean and standard deviation were calculated across all study areas: $Y_i = (X_i - \text{mean}_i) / \text{sd}_i$. This standardisation renders all biomarkers dimensionless and comparable regardless of their original units of measurement, removing the need for prior log-transformation. Finally, to ensure all values were non-negative, as required for the computation of the polygon area in the radar chart, a shift was applied: $Z_i = Y_i - \min(Y_i)$. The IBR score for each area was then calculated as the sum of the areas of the triangles formed between consecutive biomarker axes in the spider plot, following the geometric formula: $\text{IBR} = \sum 0.5 \times |Z_i \times Z_{i+1} \times \sin(2\pi/n)|$, where n is the total number of biomarkers. Higher IBR values indicate a greater overall deviation from the mean biological condition across areas, reflecting a higher level of physiological alteration.

3. Results and discussion

3.1. Biomarker responses

3.1.1. Immunotoxicity

The complement system, being a major component of the innate immunity, plays a central role in host defence. Its activation, triggered by pathogens or tissue damage, promotes pathogen opsonization, recruitment of inflammatory cells, and direct killing of foreign cells

(French et al., 2010; Berhane, 2016). In addition, immune functions are known to be sensitive to different environmental factors (Audet et al., 2016; Jara-Carrasco et al., 2015; Strandin et al., 2018; Tasnim et al., 2021; Becker et al., 2020; Ziegler et al., 2021; Sebastiano et al., 2022a; Hammouda et al., 2024). Variations in complement activity may provide useful information about individuals' physiological condition. The complement system activity measured in nestlings from agricultural area (agri 2) was significantly higher than that recorded in nestlings from wood ($p = 0.009$) and agri 1 ($p = 0.006$) areas (Fig. 1A, Table S2), suggesting a greater activation of this component of innate immunity compared with nestlings from the other study areas. The low complement system values registered in wood and agri 1 areas may reflect a partial impairment of this immune component that could be related to multiple stressors, including environmental contamination. Some papers showed a negative correlation between lysis scores (=complement system activity) and lead concentration in great tit nestlings (Vermeulen et al., 2015). Moreover, lower complement system activity has been observed in common kestrel nestlings from urban sites compared with those from less human-impacted environments (Wemer et al., 2021). Because contaminant concentrations were not measured in the nestlings of the present study, it is not possible to establish a clear causal link between biomarker responses and specific contaminants. However, recent analyses conducted on abandoned and unhatched great tit eggs collected from the same study areas in the same breeding seasons (2021–2022) showed higher PFOS concentrations and the highest concentrations of organochlorine pesticides, particularly DDT metabolites in agri 1, and higher levels of some longPFCAs in wood area (Colomer-Vidal et al., 2025). Contaminant exposure could be a possible

explanation for the lower complement activity observed in the wood and agri 1 areas, but the observed variations could also reflect the combination of multiple environmental and physiological processes. In this context, the elevated complement activity observed in the agri 2 area could not necessarily indicate a better health status, but rather a compensatory activation of innate immune defences in response to moderate environmental challenges, which may be interpreted within a hormetic framework (Costantini et al., 2010; Sebastiano et al., 2022b). Specifically, while this level of stimulation triggers an up-regulation of the system to cope with mild local pressures, the specific environmental features of agri 1 and wood area might exceed this threshold, leading to the observed lower complement activity. Another possible explanation is that individuals from the agri 2 may have experienced a higher exposure to pathogens, which could have contributed to increased activation of the complement system, whereas the lower activity observed in the wood and agri 1 areas may reflect a lower pathogen pressure. This interpretation aligns with the complement system's primary role in innate immunity, where it activates rapidly against invading microorganisms, but it is not supported by any clear evidence of parasite presence observed during handling or blood smear analysis of great tits. In fact, no parasites were noticed on the birds' surface, or during the examination of blood smears from agri 2 site. More broadly, differences in complement activity could also be influenced by ecological factors not directly related to contaminant exposure or pathogen pressure. In birds, immune responses may be influenced by confounding variables such as food availability and quality, nutritional condition, individual quality, parental provisioning, habitat characteristics, microclimatic conditions and other local environmental features

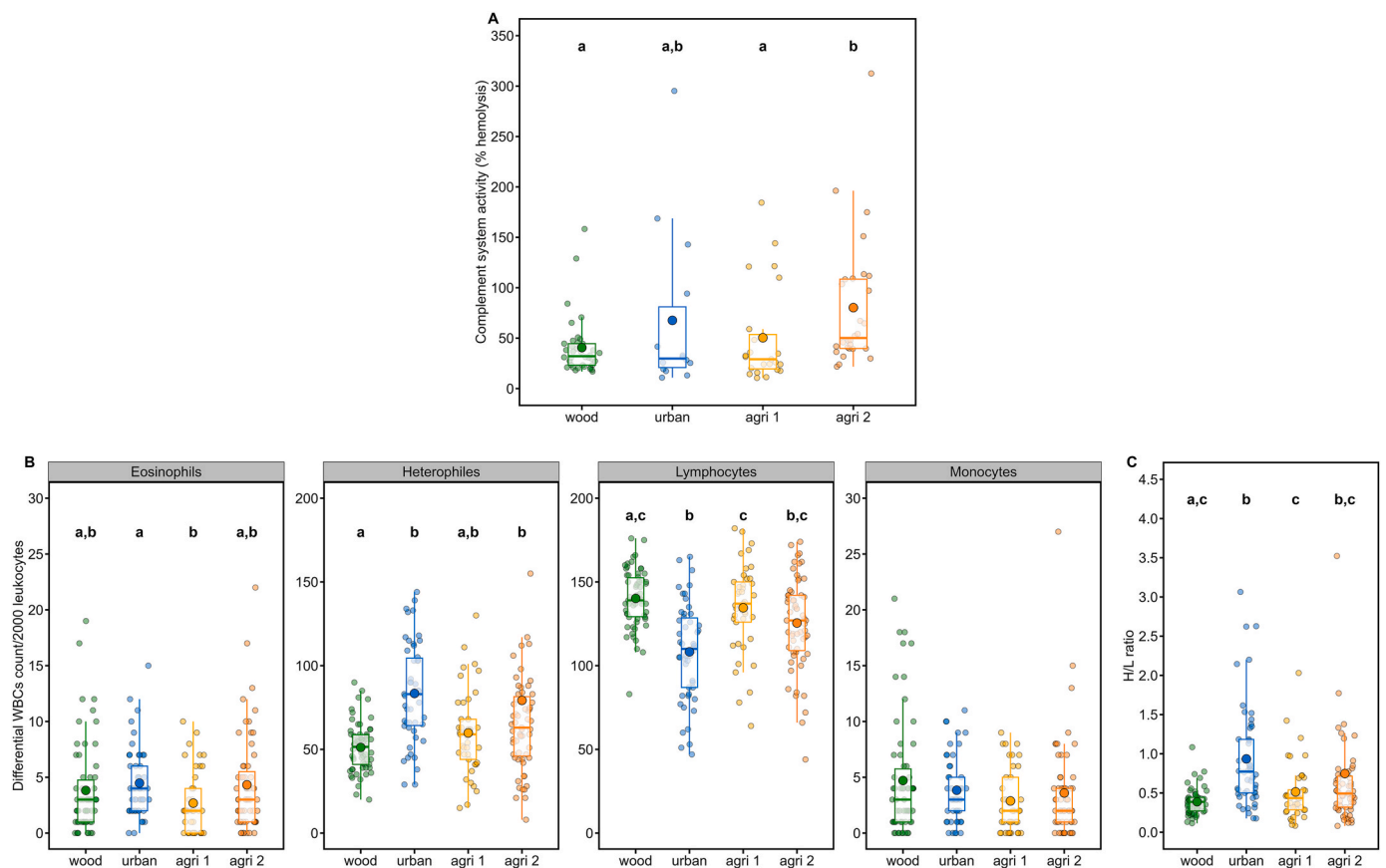


Fig. 1. Complement system activity (A), differential WBCs count (B) and H/L ratio (C) measured on blood of great tits nestlings sampled in different study areas during the breeding seasons 2021 and 2022. Box-plots represent the non-transformed raw data: the thick black lines represent the medians; the boxes encompass the interquartile ranges and the whiskers extend to the most extreme data points within $1.5 \times$ the interquartile range outside the box. Dots represent the raw data points. The mean is represented by a larger, color-filled dot. Different letters indicate statistically significant differences among areas ($p < 0.05$). (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article)

(Valdebenito et al., 2021). For example, microclimatic temperature fluctuations can significantly alter nestling immune function, while intrinsic traits like sex can modulate baseline immune responses across siblings (Skwarska, 2018). Consequently, some of the differences observed among areas may reflect the synergistic or additive stressors rather than the effects of a single environmental pressure. Although the underlying mechanisms remain uncertain, the results support the usefulness of complement activity as a biomarker of physiological variation in free-living birds exposed to contrasting environmental conditions.

Great tits from our different study areas did not show any significant differences in the respiratory burst process (Fig. S2, Table S2). To the best of our knowledge, no other studies have applied our method to evaluate the respiratory burst process in the great tit. However, our results are in line with those reported by Rainio et al. (2015) who found no relationship between phagocytic activity and lead concentrations in nestling great tits living near a metal smelter, and Giovanetti et al. (2024), in common kestrel (*Falco tinnunculus*) nestlings from contrasting environments. Conversely, Hansen et al. (2020) observed a negative correlation between organohalogenated contaminants (PCBs, DDTs, and PFAS) and leukocyte coping capacity in wild white-tailed eagle (*Haliaeetus albicilla*) nestlings. The lack of significant differences observed may indicate that this component of innate immunity was not markedly affected across the investigated areas by environmental contaminants. However, this result should be interpreted with caution, as the absence of detectable variation does not necessarily imply the absence of biological effects. Rather, it could reflect the limited sensitivity of this test to detect sub-lethal physiological alterations under field conditions, where environmental heterogeneity and natural biological variability can mask weak responses. In fact, field studies possess intrinsic limitations that need to be considered in monitoring programs, such as environmental variability, mixed and uncontrolled exposure to multiple stressors, temporal variation, and the subsequent difficulty of establishing causal relationships. Further studies are certainly needed to establish baseline or reference values, allowing for a more structured interpretation of such a complex response.

Regarding the WBCs count (Fig. 1B–Table S2), the number of heterophiles was significantly higher in nestlings from urban ($p = 0.015$) and agri 2 ($p = 0.016$) areas compared to wood one. Eosinophils were significantly more numerous in urban area than agri 1 ($p = 0.014$) and basophil cells were not found in any of the analysed samples. Lymphocytes were significantly less abundant in urban nestlings than those from wood ($p = 0.001$) and agri 1 ($p = 0.004$) areas and monocytes did not differ among study areas. The differential WBC count provides an indication of potential infectious or inflammatory conditions in animals (Campbell, 1995) and can also reveal possible alterations in immune function. Urban environments are typically characterized by the simultaneous occurrence of multiple stressors, including chemical contamination, light and noise pollution, human disturbance, altered food availability and quality and pathogen exposure (Santos et al., 2025; Isaksson, 2018). Such environmental pressures may influence immune function and contribute to alterations in leukocyte profile (Lunda Monassa et al., 2026). The high values of heterophils combined with low lymphocyte values (H/L ratio) in the blood of great tit from the urban area resulted significantly higher than those from wood ($p = 0.019$) and agri 1 ($p = 0.004$) areas (Fig. 1C–Table S2). This parameter is widely used as an indicator of chronic physiological stress in wild birds because it tends to increase in response to stressors (Davis et al., 2008; Latimer et al., 2020) and its variation offer useful information on individual conditions and habitat quality (Cid et al., 2018; Skwarska, 2018; Ancora et al., 2025). Our results are consistent with previous studies reporting associations between contaminant exposure and leukocyte profile alterations in great tits. Bauerová et al. (2020) showed that H/L ratio increased with increasing blood Pb concentrations in a suburban population of great tits monitored over twelve years, while Bauerová et al. (2017) reported evidence of long-term stress in urban great tit populations, where higher H/L ratios were associated with increasing

feather heavy metal contamination. Similarly, the H/L ratio recorded in our urban nestlings was almost four times higher than values reported for Eurasian tree sparrows (*Passer montanus*) inhabiting highly polluted Chinese areas (Li et al., 2021). Taken together, these findings suggest that urban stressors, including potential contaminant exposure, may have contributed to the altered leukocyte profile observed in the urban area. Moreover, the H/L ratio was negatively correlated with body mass ($p < 0.001$, Table S2), suggesting that nestlings in poor physical condition were less responsive to external pressures. When facing environmental challenges, individuals in better physical condition may be able to allocate resources more efficiently among growth, maintenance, and energetically demanding immune responses. Then, in addition to potential environmental contamination, the high levels of H/L ratio in our urban nestlings may also reflect the physiological costs associated with urban living. For example, dietary quality may represent an important component of these costs. Beyond food quantity, the nutritional composition of available resources can influence physiological condition by determining the availability of energy and key nutrients required to sustain growth, immune function and other physiological processes. Consequently, reduced access to high-quality prey may limit the resources available for immune responses and the maintenance of oxidative balance. In great tits, variation in food quality and resource availability has been shown to influence both body condition and immune responsiveness (Bailly et al., 2016). Furthermore, habitat fragmentation may increase foraging effort, while urban heat island effects may increase thermoregulatory demands. Together with the energetic costs associated with detoxification processes, these factors may influence individual condition and immune function (Chatelain et al., 2026). We can therefore hypothesize that the great tits living in the urban area were subjected to conditions promoting chronic physiological stress and/or inflammation (Campbell & Ellis, 2007). Conversely, we should also consider the possibility that these elevated H/L ratios reflect a form of physiological plasticity. In a resilient and adaptable species like the great tit (Isaksson, 2018), this dynamic cellular adjustment may enhance physiological flexibility, helping individuals to optimize their response to environmental perturbations and fluctuations. Therefore, this mechanism could represent an adaptive strategy typical of animals living in urban environments. In these contexts, heterophil levels may increase to enhance the organisms' capacity to combat infections, while the reduction in lymphocytes serves to limit the activation of the adaptive immune response (Goessling et al., 2015; Ribeiro et al., 2022).

3.1.2. Oxidative status

The highest levels of TAS were recorded in the agri 1 area with a significant difference with respect to urban area ($p = 0.003$) that instead showed the lowest values. No statistical differences were observed between the two agricultural areas (Fig. 2, Table S3). The highest concentration of antioxidants measured in nestlings from agri 1 may suggest that these birds are subjected to some environmental pressures. The TAS assay evaluates the non-enzymatic antioxidant levels of dietary and endogenous sources (Bourgeon et al., 2012). Different human threats, such as human presence, light and noise pollution, contaminants, climate change, food intake and infectious diseases (Beaulieu and Costantini, 2014) can act on the oxidative status of wild animals. The increase of antioxidants, as a protective response to the rising ROS production, has been reported for some contaminants including heavy metal, polybrominated, diphenyl ethers (PBDEs) and organochlorine pesticides (OCPs) (Espín et al., 2014; Espín et al., 2016; Abbasi et al., 2017; Bourgeon et al., 2012) on different avian species. The agri 1 area, Oasi Ca' di Mezzo, is an artificial engineered phytodepuration site, surrounded by extensive wheat and maize cultivation. This site collects the wastewater of a canal that drains water from upstream farmland created to remove nitrogen and phosphorus before water is released into Venice lagoon. Moreover, the Bacchiglione River also borders this area and it is nearby the Brenta River, two streams contaminated, over the years, by different substances such as PFAS (Sciancalepore et al., 2021;

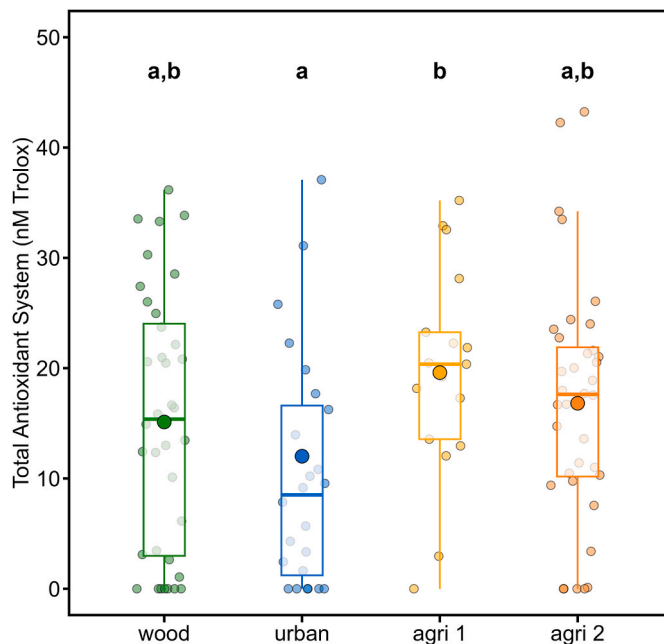


Fig. 2. TAS measured in the blood of great tit (*Parus major*) nestlings sampled in different study areas during the breeding seasons 2021 and 2022. Box-plots represent the non-transformed raw data: the thick black lines represent the medians; the boxes encompass the interquartile ranges and the whiskers extend to the most extreme data points within $1.5 \times$ the interquartile range outside the box. Dots represent the raw data points. The mean is represented by a larger, color-filled dot. Different letters indicate statistically significant differences among areas ($p < 0.05$). (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article)

Valsecchi et al., 2015), herbicides (Masiol et al., 2018) and by other compounds (Miranda et al., 2021; Giacometti & Perin, 2016). Based on the environmental characteristics of this area, we hypothesize that the complex mixture of local anthropogenic stressors might induce an up-regulation of antioxidants in nestlings. Although we did not evaluate contamination directly in the nestlings, high levels of PFAS and OCs were found in abandoned and unhatched great tit eggs from the same area in our study (Colomer-Vidal et al., 2025), suggesting that chemical exposure may be one of the environmental pressures acting on these animals. Nonetheless, it must be taken into account that other confounding factors may have influenced these values. Since the TAS assay measures non-enzymatic antioxidants, dietary intake and habitat-specific food quality can play a key role, as variations in the availability of antioxidant-rich prey and in the overall composition of the diet are known to affect circulating antioxidant capacity in birds (Cohen et al., 2007; Cohen et al., 2009; Romero-Haro & Alonso-Alvarez, 2014). Furthermore, intrinsic factors, including individual body condition, slight differences in hatching dates, parental oxidative status, may contribute to shaping baseline antioxidant levels independently of chemical pressures (López-Arrabé et al., 2016; Salmón et al., 2018), in line with the idea that oxidative status reflects a complex interplay between individual physiology, life-history stage and environmental conditions (Costantini, 2019). Moreover, the low TAS levels measured in our urban nestlings may reflect a struggle to cope with pro-oxidative conditions induced by either urban stressors or inflammatory processes. Although our results align with previous studies that reported a decrease in TAS in urban great tits (Herrera-Dueñas et al., 2014, 2017; Kurhaluk et al., 2022), this trend should be interpreted with caution, given the lack of direct evidence for oxidative damage. Indeed, it is also plausible that city birds down-regulate antioxidant levels as a physiological strategy to face this anthropized environment (Costantini et al., 2014). Taken together, these variations in TAS underscore that

evaluating antioxidant defenses alone offers only a partial view. Future studies should include biomarkers of oxidative damage to gain a more comprehensive understanding of variations in oxidative status.

3.1.3. Genotoxicity biomarkers

No significant differences were shown for great tit nestlings from the different study areas for the comet assay (Fig. S3, Table S4). To date, since this test has not been applied before on our species, no basal values are useable as references. Comparing our data with those available in the literature on other Passeriformes, the DNA fragmentation levels detected in the different areas are lower than those measured in free-living spotless starlings *Sturnus unicolor* living in a site located near a Spanish-protected area (27 % of tail DNA) (Montoya et al., 2020). In addition, different species of birds sampled in contaminated areas showed higher values of comet assay (Sicol et al., 2010; Bonisoli-Alquati et al., 2010; Galván et al., 2014; de Freitas et al., 2024). It is crucial to note that the average DNA damage value of approximately 14% recorded in nestlings across our four sites aligns with international guidelines for genotoxicity testing, where similar levels are considered baseline values (Lovell & Omori, 2008). However, 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.

The number of bud and notched nuclei, as well as total abnormalities counted in nestlings from urban, agri 1 and agri 2 areas were significantly higher than those of the wood area, with the urban site showing the highest values ($p < 0.006$; Fig. 3A and B, Table S4). Segmented nuclei were significantly more abundant in agri 1 than in urban ($p = 0.032$). Kidney nuclei values were significantly higher in pullets from agri 1 ($p = 0.003$) and agri 2 ($p < 0.001$) than those from wood area; moreover, a significant difference also emerged between agri 2 and urban area ($p = 0.013$). Finally, the number of micronuclei was significantly higher in great tits from the urban ($p = 0.040$) and agri 1 ($p = 0.007$) areas than those from wood area (Fig. 3B–Table S4). It should be underlined that bud nuclei were the predominant abnormality among those counted. Environmental contaminants, such as heavy metals, pesticides, PAHs and POPs in general, can affect DNA integrity causing genomic alterations in the erythrocyte nucleus (Louzon et al., 2019) of avian species (Gill et al., 2018; Cocco, 2022; Ceyca-Contreras et al., 2020), which are ultimately expressed as erythrocytic nuclear abnormalities. These abnormalities are generally induced either directly by pollutants or indirectly through oxidative stress. The resulting overproduction of ROS can subsequently damage key macromolecules, including proteins, lipids and DNA (Espín et al., 2014). The abnormality frequencies measured in our areas are higher than the values observed in other contaminated areas (Tomazelli et al., 2022; Frixione et al., 2022; Stocker et al., 2024; de Freitas et al., 2024). Conversely, Drahulian et al. (2018) showed a micronuclei frequency ($3.4 \pm 0.45\%$) higher than that of great tits from our study. Quero et al. (2016) found MN frequencies in different passeriformes from a biosphere reserve in Argentina that were in line with the values recorded in our urban and agri 1 areas. However, the presence of MN that we found in urban birds was higher than the reference values (spontaneous MN: $0.2/2000$ erythrocytes) suggested for adult passeriformes (Zúñiga-González et al., 2001). Thus, a comparison with the literature might lead us to suppose the influence of extrinsic variables, such as the exposure to genotoxic compounds, in urban and agri 1 areas, especially given the typical responsiveness of this biomarker to genotoxic agents. Concurrently, the fluctuations of oxidative status and immune parameters, naturally subject to various ecological factors, help outline a broader scenario of physiological responses to anthropogenic stressors in nestlings inhabiting more anthropized sites.

3.1.4. Neurotoxicity biomarkers

BChE and CaE activities did not show significant differences among study areas (Fig. S4, Table S5). BChE catalyses the hydrolysis of the

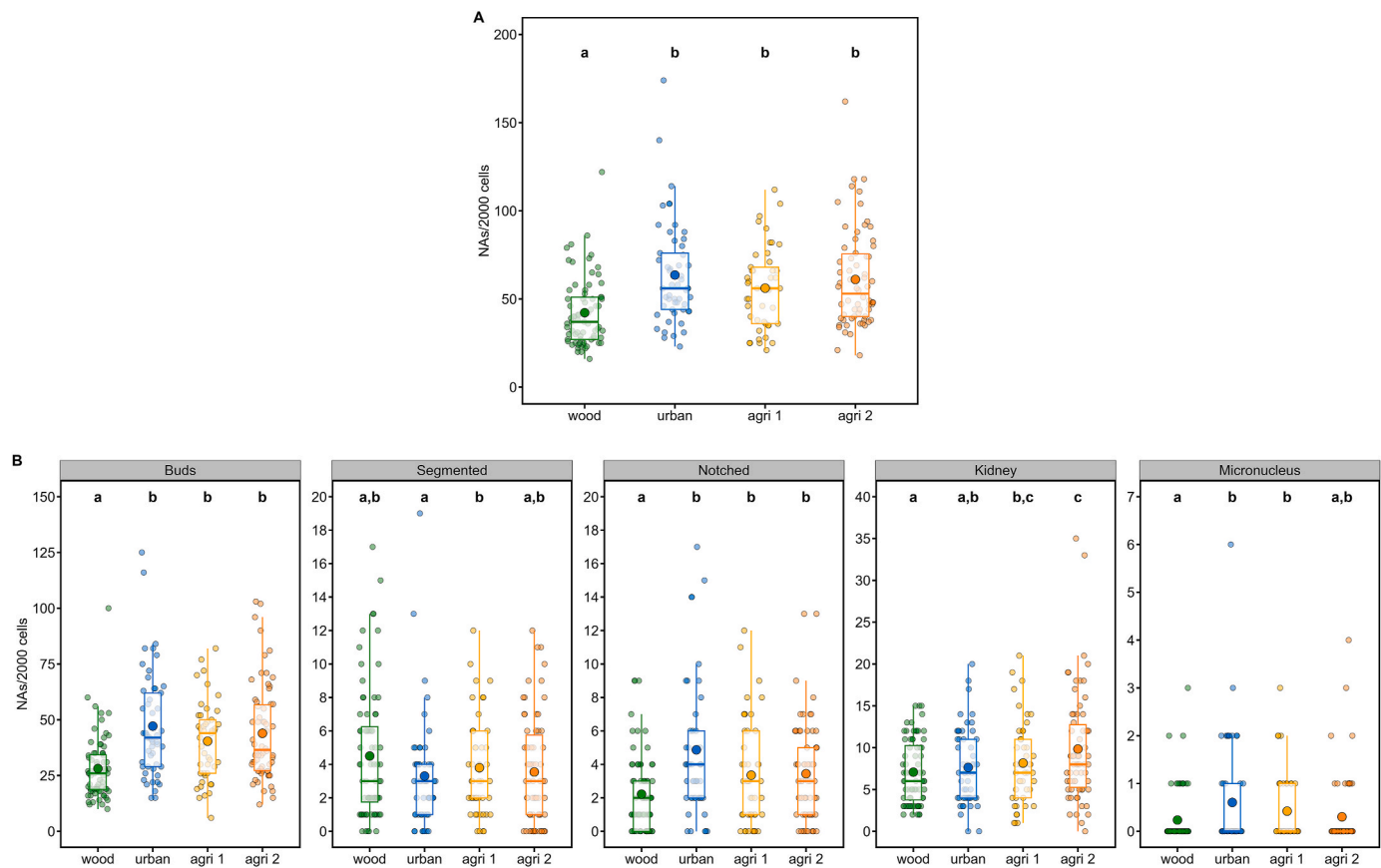


Fig. 3. Total erythrocytes nuclear abnormalities (A), erythrocyte nuclear abnormalities divided by nucleus shape (B) measured in blood of great tits (*Parus major*) nestlings sampled in different study areas during the breeding seasons 2021 and 2022. Box-plots represent the non-transformed raw data: the thick black lines represent the medians; the boxes encompass the interquartile ranges and the whiskers extend to the most extreme data points within $1.5 \times$ the interquartile range outside the box. Dots represent the raw data points. The mean is represented by a larger, color-filled dot. Different letters indicate statistically significant differences among areas ($p < 0.05$). (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article)

neurotransmitter acetylcholine (Monteiro et al., 2005; Radic and Taylor, 2006); BChE is also involved in preventing the inhibition of acetylcholinesterase (AChE), thus it is considered a system of protection for this enzyme (Omedes et al., 2023). CaEs facilitate the excretion of polar chemicals, and simultaneously protect AChE by providing alternative sites for binding insecticides (Casey Laizure et al., 2013; Arufe et al., 2010). Organophosphate (OPs) and carbamate (CBs) pesticides can bind to esterases, causing their inhibition in the birds (Santos et al., 2012; Narváez et al., 2016). In addition, other contaminants such as metals, detergents and PAHs have been found to generate similar inhibitory effects (Casini et al., 2006; Santos et al., 2012; Bjedov et al., 2023; Nos et al., 2024). The mean values of BChE recorded in the blood of great tits individuals from all the study areas were higher than those found by Norte et al. (2008a, 2008b) in adult and nestling great tits living in woodlands, suburban or industrial areas. Our BChE data were also twice than those reported by Tsarpali et al. (2020) in specimens of free-living Eleonora's falcons *Falco eleonora*, while were in line with values of native Australian birds (e.g. *Melopsittacus undulatus*, *Taeniopygia bichenovii* and *Rhipidura leucophrys*) (Strum et al., 2008). Regarding CaE, we recorded values higher than those reported by Giovanetti et al. (2024) and Nos et al. (2024) in *Falco tinnunculus* and *Larus michahellis* sampled in urban areas, respectively. Similarly to our findings, Norte et al. (2008a, 2008b) did not detect significant differences in BChE activity among birds inhabiting areas with different anthropogenic impacts. The lack of spatial variation may suggest that this enzyme is unresponsive to broad habitat-related differences, but rather to exposure to specific contaminants. Therefore, the use of BChE as a biomarker may be more informative in targeted exposure scenarios,

particularly when organisms are exposed to compounds known to affect cholinesterase activity.

3.1.5. Porphyrins

Coproporphyrins showed the highest levels in wood area compared to agri 1 area ($p = 0.025$); protoporphyrins level of wood was significantly higher than that measured in great tits from the agri 2 area ($p = 0.042$). Total porphyrin levels were higher in wood area, with a statistically significant difference relative to both agricultural areas (agri1, $p = 0.042$; agri2, $p = 0.019$; Fig. 4, Table S6). The fundamental physiological role of porphyrins is linked to heme synthesis, in which they represent either intermediate metabolites or oxidation byproducts of these intermediates. To our knowledge, no published studies are available on porphyrin levels in excreta of this species. Levels of total porphyrins measured in our nestlings are higher than those found by Casini et al. (2001) in excreta of *Pelecanus thagus*, *Phalacrocorax olivaceus* and *Larus dominicanus* from Chilean industrial (8600 pmol/g d. w.) and agricultural (6600 pmol/g d. w.) areas and lower than those recorded by Sicolo et al. (2009) in urban (about 52000 pmol/g d. w.) *Columbia livia* excreta. In line with Casini et al. (2001) results, we also found the predominance of protoporphyrins with respect to the other analysed classes of porphyrins. Our protoporphyrin values were about three times higher than those recorded in Humboldt penguins *Spheniscus humboldti* living near an industrialized area (Celis et al., 2014). We hypothesize that air pollutants from vehicular traffic and home heating originating from the nearby city of Padua may have significantly affected this wooded environment. Lead (Pb), present in highly trafficked areas, has been shown to induce the accumulation of protoporphyrins, as reported by

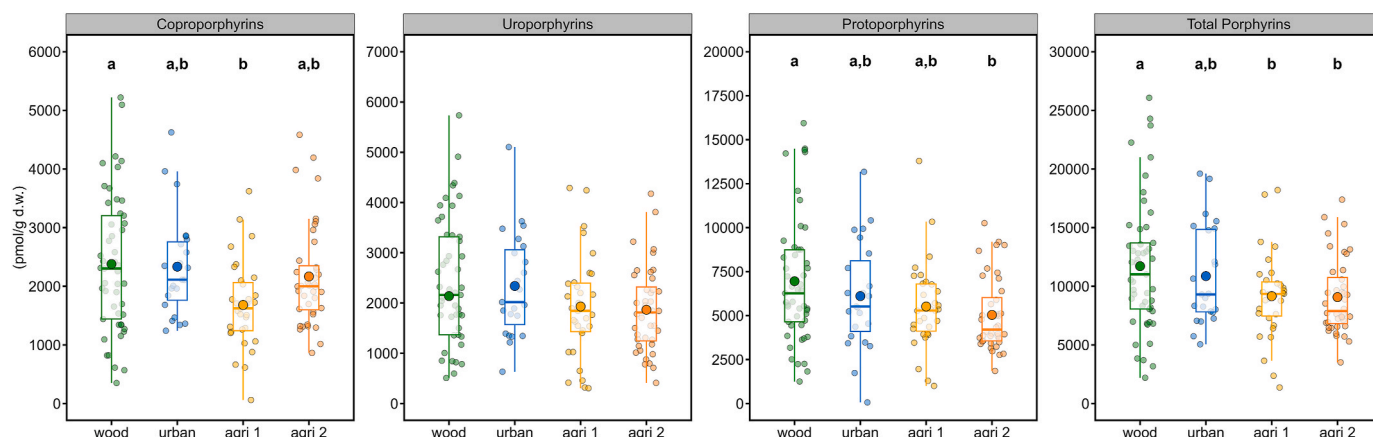


Fig. 4. Copro-, uro-, protoporphyrins and total porphyrins measured in excreta of great tit (*Parus major*) nestlings sampled in different study areas during the breeding seasons 2021 and 2022. Box-plots represent the non-transformed raw data: the thick black lines represent the medians; the boxes encompass the inter-quartile ranges and the whiskers extend to the extreme data points within $1.5 \times$ the interquartile range outside the box. Dots represent the raw data points. The mean is represented by a larger, color-filled dot. Different letters indicate statistically significant differences among areas ($p < 0.05$). (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article)

Casini et al. (2003). Moreover, Padua, located about 15 km away, has been recognized as one of the most polluted cities in Europe by the European Environment Agency (Thunis et al., 2017), primarily because of particulate matter (PM) pollution caused by combustion processes and traffic-related emissions (Tateo et al., 2019). Supporting this hypothesis, Scartezzini et al. (2021) found that the mean concentration of PM_{2.5} in Euganean Hills was similar to those of Padua centre. Crucially, Sicolo et al. (2009) found a close connection between increasing concentrations of protoporphyrins and PM_{2.5} in individuals of *Columba livia* sampled in Milan. Therefore, great tit nestlings from the wood area may be exposed to particulate air contamination originating from the surrounding zones or even from distant regions through long-range atmospheric transport. Additionally, high levels of PFAS (specifically PFUnDA and PFTrDA) indicative of atmospheric deposition, were recorded in abandoned and unhatched eggs of our great tits collected in the same campaign in the wood area (Colomer-Vidal et al., 2025). LongPFCA precursors can undergo long-range atmospheric transport and accumulate in forest environment, where tree canopies efficiently capture these highly volatile compounds. Following litterfall and decomposition, long-chain PFCA precursors may become available in forest soils and can be assumed by great tits via the ingestion of soil organisms, such as earthworms, which represent a key food source for these birds. These compounds might also contribute to the increase in porphyrin levels, although to date no studies investigated the correlation between PFAS and porphyrins. The suspected presence of airborne contaminants could be a potential limitation when considering this study area as a true reference site. Nevertheless, within the highly anthropized context of this region, this site might still be considered the least impacted among those investigated. Indeed, while it does not serve as a conventional control site, as obtaining an absolute and entirely pristine reference is notoriously challenging in field studies, it remains clearly distinct from the other areas by being free from direct and localized multi-pressures. Therefore, despite these potential limitations, the wood area could still offer a useful term of comparison to evaluate the potentially stronger and more direct anthropogenic impacts occurring in more anthropized environments, such as urban and agricultural ones. Even more so, this result highlights, once again, the importance of employing a multi-biomarker approach to highlight biological responses that may differ in both type and intensity across contrasting and inherently different environments.

3.1.6. Integrated Biological Response (IBR)

The results of the Integrated Biological Response (IBR) for each

sampling area are shown in Fig. 5. The urban area exhibited the highest IBR value (5.25), with respiratory burst, the H/L ratio, total ENA, and the comet assay acting as the most discriminant factors for this group. The agri 1 and agri 2 areas showed similar IBR values (3.2 and 2.2, respectively). Specifically, variations in BChE and complement system activities, TAS levels and genotoxicity biomarkers (comet assay and total ENA) were the most relevant responses that explain the IBR index for agri 1. Meanwhile, TAS, the H/L ratio, and total ENA were the most discriminant factors for agri 2. The wood area presented the lowest IBR value (1.46), although variations were observed in total porphyrins and CaE. In this study, we used the IBR to clearly visualize which biomarkers were the most sensitive in contrasting habitats, providing a comprehensive synthesis of the overall biological responses. The spatial arrangement of these biomarkers in the spider plot allowed for an integrative perspective across molecular and cellular biomarkers (Vieira et al., 2014). Based on the IBR score, the urban and agricultural areas were identified as the sites under the highest physiological stress, probably due to the diverse anthropogenic pressures present in these environments. Among the two agricultural areas, agri 1 showed major alterations across the different parameters, particularly oxidative status, immune system responses, and DNA damage. Moreover, the index highlighted that different biomarkers reflect distinct biological processes, operate at different levels of organization, and may vary in their sensitivity, response time, and recovery dynamics. In our study, some endpoints proved to be more sensitive than others in detecting physiological alterations associated with multiple stressors, which are not expected to affect all endpoints in the same way or with the same intensity. This variability highlights the complexity of physiological responses under field conditions; in fact, heterogeneous and uncontrolled exposure scenarios, where multiple stressors act simultaneously and interact with natural sources of biological variability, inherently characterize field studies. Factors such as diet, habitat characteristics, individual condition, and temporal variation may contribute to shaping biomarker responses and potentially mask subtle effects. In addition, wild birds may exhibit a considerable degree of physiological plasticity, enabling them to compensate for or tolerate moderate environmental challenges without showing uniform alterations across all biomarkers. Therefore, the heterogeneous responses observed across biomarkers support the need for a multi-biomarker approach, as no single endpoint is able to capture the complexity of an organism's biological responses to changing environments. Our findings highlight that no single endpoint can capture the complexity of wildlife responses to environmental changes. The integration of complementary biomarkers through the IBR index

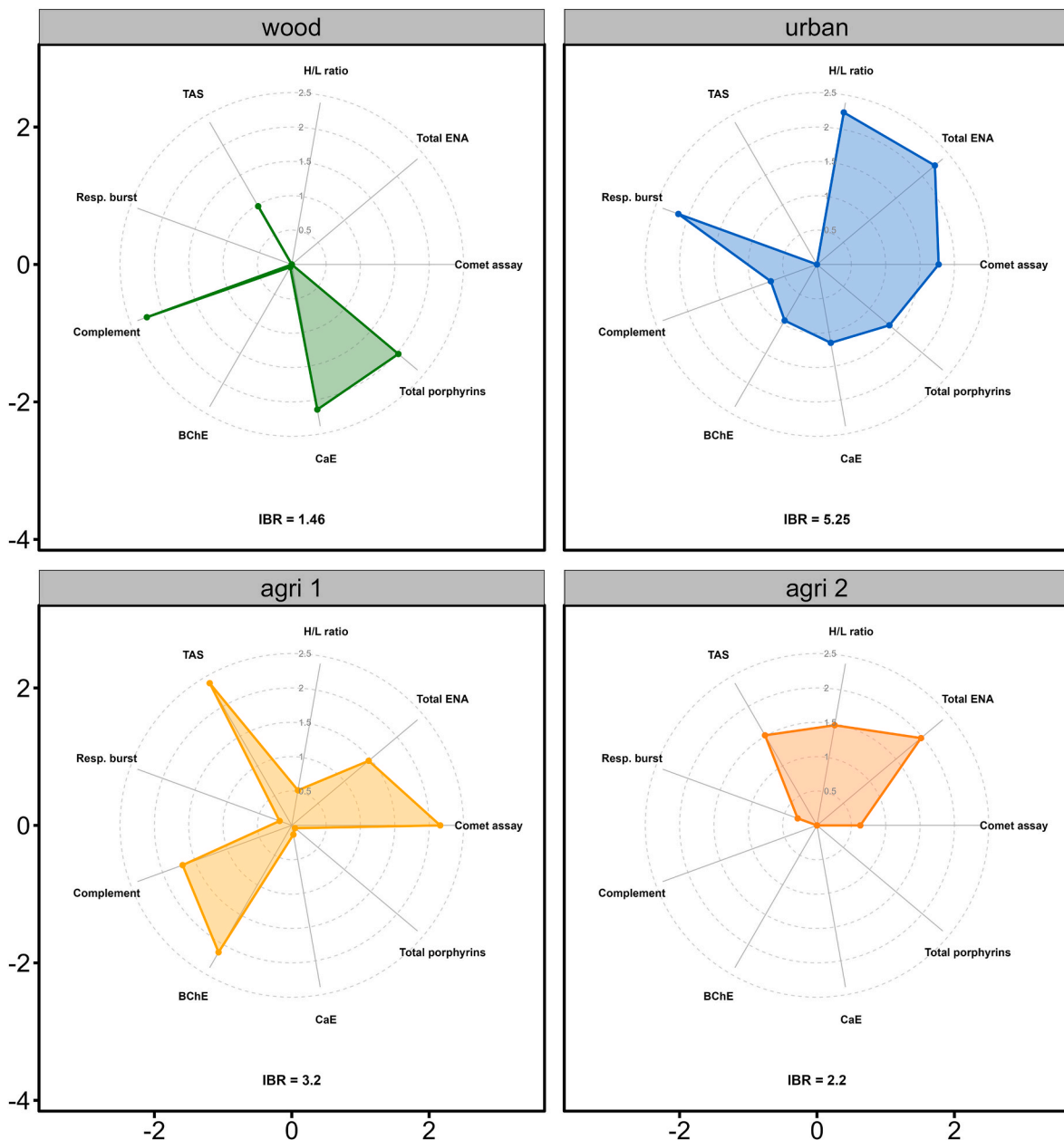


Fig. 5. Spider plot of the Integrated Biomarker Response (IBR) index.

provides an effective, ecologically relevant tool for environmental monitoring, offering valuable support for avian conservation strategies and regulatory policies.

4. Conclusions

This study allowed acquiring new knowledge on the ecotoxicological status of great tit populations from areas with different anthropogenic impacts (wood, urban and agricultural) by applying a multi-biomarker approach based on cellular and molecular responses. We found that populations of urban and agricultural areas were those with the most significant impacts resulting from contamination and other possible pressures typical of anthropized environments; these findings are also supported by the IBR index results. In fact, the IBR data fully aligned with and confirmed the trends observed through the individual biological responses, demonstrating how integrated indices mirror discrete cellular alterations. Unlike traditional monitoring focused on isolated parameters, this multi-biomarker approach provides a comprehensive,

integrated overview of avian toxicological health. It proved to be a highly effective field tool, capable of capturing site-specific physiological costs triggered by overlapping environmental stressors. Crucially, the application of the IBR index served as a valid tool to highlight the overall trend of physiological stress, successfully synthesizing heterogeneous biomarker responses into a complete representation of cumulative environmental pressures across contrasting habitats. This approach can offer a more comprehensive understanding of the ecological impacts of anthropogenic stressors on wildlife populations over time. As such, it holds potential for application at managerial and regulatory levels to support the conservation and protection of wild and endangered bird species. Specifically, this methodology can help identify critical environmental conditions affecting wildlife and serve as a valuable resource for policymakers in evaluating the effectiveness of conservation regulations and strategies for managing threatened species. In future studies, combining this multi-biomarker framework with population dynamics and detailed chemical profiles could fully unravel the subtle interactions between anthropogenic pressures and natural

biological variability, establishing an ecologically relevant framework for modern environmental monitoring.

CRedit authorship contribution statement

Laura Giovanetti: Writing – review & editing, Writing – original draft, Resources, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Silvia Casini:** Writing – review & editing, Supervision, Conceptualization. **Matteo Beccardi:** Writing – review & editing, Methodology, Investigation, Formal analysis, Data curation. **Matteo Schiavinato:** Writing – review & editing, Methodology, Investigation. **Julia S.M. Luthi:** Investigation. **Gabriele Fontanelli:** Investigation. **Andrea Franchi:** Investigation. **Stefania Ancora:** Writing – review & editing. **Flavio Monti:** Writing – review & editing. **Ilaria Caliani:** Writing – review & editing, Writing – original draft, Supervision, Project administration, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, 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.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.envpol.2026.128755>.

Data availability

Data will be made available on request.

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