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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

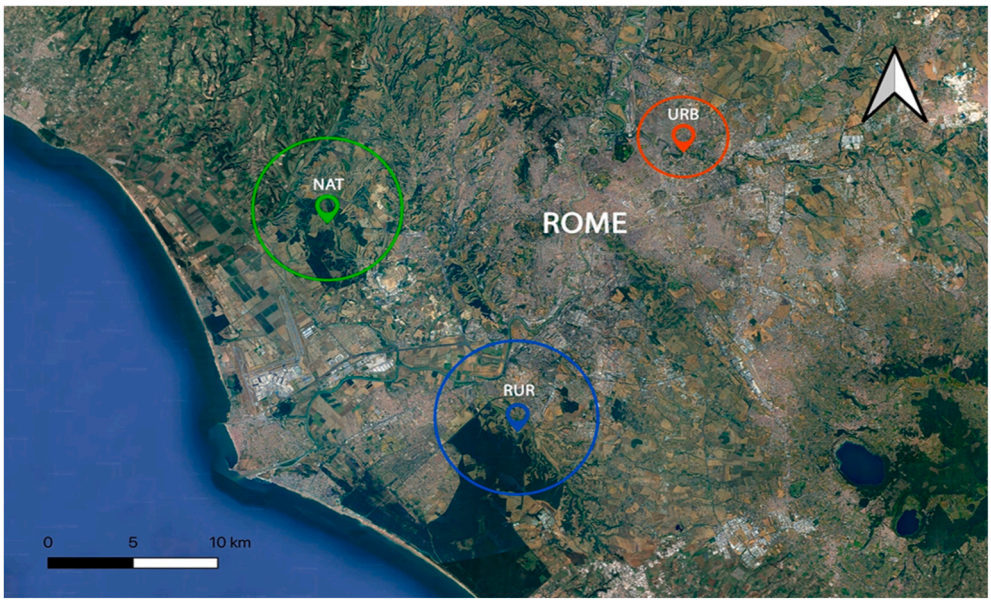

Fig. 1. Study areas of common kestrel nestlings located in the Lazio Region: NAT (green), RUR (blue) and URB (red).

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

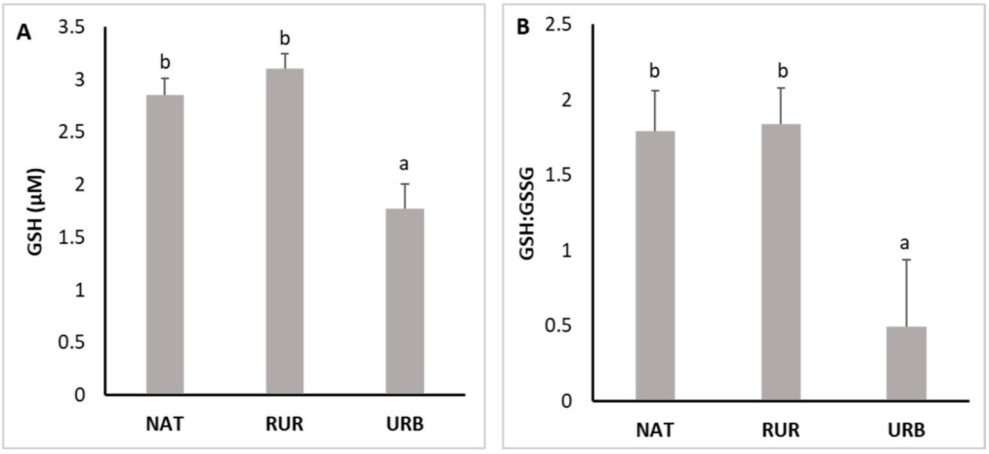

Fig. 2. (A) Plasma concentration of reduced glutathione (GSH) and (B) ratio between reduced and oxidized glutathione (GSH:GSSG) in common kestrel nestlings sampled in NAT = natural, RUR = rural, and URB = urban areas. Different letters indicate statistically significant differences ($p < 0.05$). Results 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

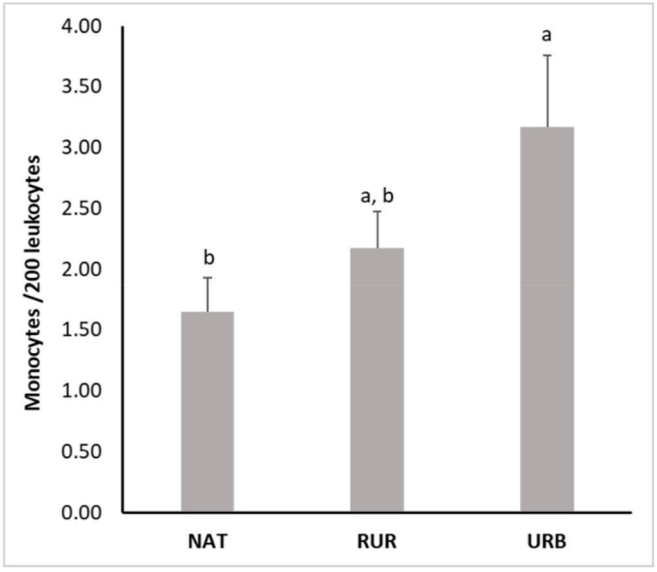

Fig. 3. Variation in the proportion of monocytes among the three study areas (NAT = natural, RUR = rural, and URB = urban) in common kestrel nestlings. Different letters indicate statistically significant differences ($p<0.05$). Results are presented as estimated marginal means ± SE (sqrt-root transformed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

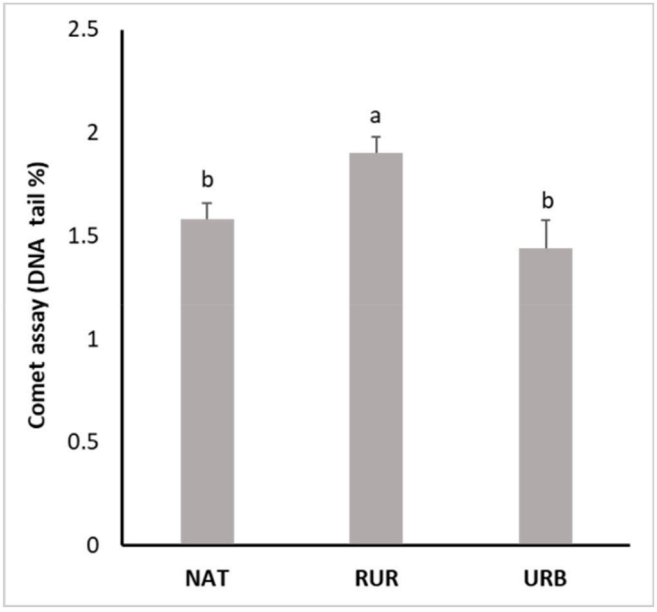

Fig. 4. Comet assay results of common kestrel nestlings sampled in NAT = natural, RUR = rural, and URB = urban areas. Different letters indicate statistically significant differences ($p<0.05$). Results are presented as estimated marginal means ± SE (sqrt-root transformed values).

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

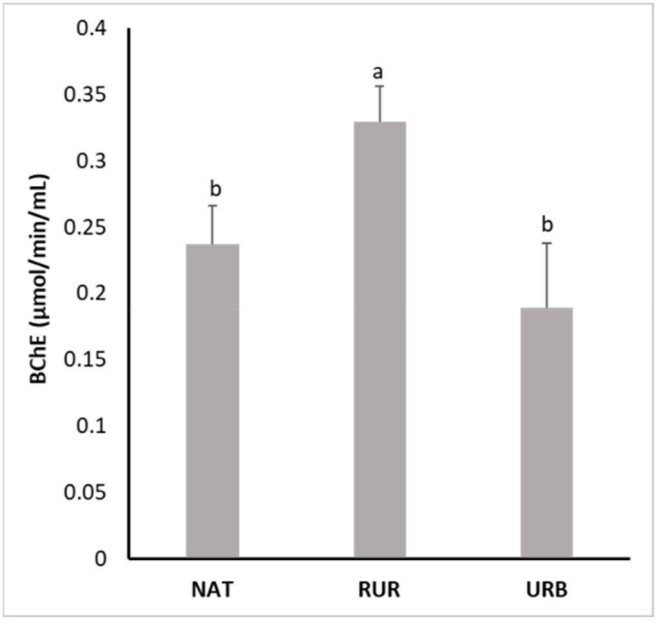

Fig. 5. Activity of butyrylcholinesterase (BChE) in common kestrel nestlings sampled in NAT = natural, RUR = rural, and URB = urban areas. Different letters indicate statistically significant differences ($p<0.05$). Results are presented as estimated marginal means ± SE.

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.

Acknowledgements

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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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