Journal Pre-proof

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PII: S0048-9697(24)02731-1

DOI: https://doi.org/10.1016/j.scitotenv.2024.172585

Reference: STOTEN 172585

To appear in: *Science of the Total Environment*

Received date: 15 January 2024

Revised date: 16 April 2024

Accepted date: 17 April 2024

Please cite this article as: G. Damiani, M. Sebastiano, G. Dell'Omo, et al., Blood transcriptome analysis of common kestrel nestlings living in urban and non-urban environments, *Science of the Total Environment* (2023), https://doi.org/10.1016/j.scitotenv.2024.172585

Blood transcriptome analysis of common kestrel nestlings living in urban and non-urban environments

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Abstract

Urbanisation is one of the main anthropogenic forms of land cover affecting the ever-increasing number of wild animals and their habitats. Physiological plasticity represents an important process through which animals can adjust to the novel conditions of anthropogenic environments. Relying on the analysis of gene expression, it is possible to identify the molecular responses to the habitat conditions and infer possible environmental factors that affect the organismal physiology. We have quantified for the first time the blood transcriptome of common kestrel (*Falco tinnunculus*) nestlings living in urban sites and compared it to the transcriptome of kestrel nestlings inhabiting rural and natural environments. We found mild differences in the expression of genes among sites, indicating adaptability or acclimation of the birds to the urban habitat. We identified 58 differentially expressed genes between urban and natural kestrels, and 12 differentially expressed genes between urban and rural kestrels. The most striking differences among sites involved inflammatory-immunological, metabolic, apoptosis, DNA repair and development genes. In particular, we found that (i) urban kestrel nestlings had higher expression of genes linked to inflammation, repair of DNA damage, or apoptosis than natural kestrel nestlings, and (ii) natural and rural kestrel nestlings had higher expression of genes linked to the development and activation of immune cells, type I interferon response, or major histocompatibility complex than urban kestrel nestlings. Finally, the KEGG enrichment analysis identified the insulin signaling as the main pathway that differed between natural and urban kestrel nestlings. This is one of a limited number of studies on vertebrates that revealed habitat-associated differences in the transcriptome. It paves the way for further in-depth studies on the links between physiological variation and habitat structure at different spatial and temporal scales.

Keywords: RNA sequencing, common kestrel, adaptation, immunology, birds.

Introduction

Urbanisation is the fastest-growing anthropogenic form of land cover (Zhang, 2016). As the human population is expected to grow further in the coming decades, the conversion of natural habitats into urban areas is also expected to increase (McGranahan and Satterthwaite, 2014). In urban areas animals rely a number of behavioural and physiological responses to be able to exploit these new environments (e.g. Partecke et al. 2006; Bonier et al. 2007; Costantini et al. 2014b; Salmón et al., 2016, 2017; Charmantier et al. 2017; Sepp et al. 2018; Murray et al., 2019). Despite some species being severely damaged by urbanisation, others have acclimatised or adapted to live in cities and other anthropogenic environments (Diamond et al., 2022).

Cities offer some potential benefits for wildlife, such as reduced fluctuations in abiotic factors (e.g. temperature) and continuous availability of food (e.g. Adams, 1994; Krimowa 2012; Campbell-Staton et al., 2020; Hansen et al., 2020). Some studies found that urban birds might have higher stress tolerance or resistance than their rural conspecifics (e.g. Partecke et al. 2006; Bonier et al. 2007; Costantini et al. 2014b; Sinclair et al. 2022). However, many other studies documented negative effects of urban environments on wildlife, such as reduced individual health, shorter telomeres, and altered population dynamics (e.g. Hendry et al., 2008; Salmón et al., 2016, 2017; Murray et al., 2019; Putman and Tippie, 2020; Thompson et al., 2022). A recent meta-analysis found a small but significant negative relationship between urbanisation and wildlife health, which was mainly driven by considerably higher exposure to pollutants

and greater parasite abundance in urban animal populations compared to conspecific populations living in less urbanised environments (Murray et al., 2019).

Birds are especially ubiquitous and abundant in urban environments (Bonier et al., 2007), thus they are particularly suitable subjects in the investigation of molecular, physiological and behavioural responses to anthropised environments. Urban populations of some bird species tend to reproduce earlier (e.g. Beck and Heinsohn, 2006) and experience a reduced reproductive success (e.g. Beck and Heinshon, 2006; Charter et al., 2007) compared to conspecific populations that live in natural environments. Some studies suggested that this differentiation between conspecific populations living in different environments reflects phenotypic responses (plasticity) to anthropogenic environments (Garroway and Sheldon, 2013). By contrast, other studies suggested that micro-evolutionary processes can also generate genetic divergence between populations (e.g. Perrier et al., 2018; Putman and Tippie, 2020). For example, Isaksson (2015) found that single nucleotide polymorphisms (SNP) in urban birds are associated with a higher tolerance to toxic substances.

Birds of prey are particularly susceptible to changes in the environment due to their position at the top of the food chain, yet some species are increasingly associated with urban environments (Kettel et al., 2018). For example, some studies suggested that the peregrine falcon (*Falco peregrinus*) is an urban exploiter because the reproductive success of its urban populations is higher than that of non-urban populations (e.g. Kettel et al., 2018, 2019). By contrast, other birds of prey, like the common kestrel (*Falco tinnunculus*), tend to show a reduced reproductive performance in urban areas (Kettel et al., 2018), possibly due to reduced food quality (Sumasgutner et al., 2014a,b). However, other studies showed that even non-urban habitats can present various challenges for

common kestrels, such as increased predation risk (Petty et al. 2003), habitat homogenisation (Costantini et al., 2014a), exposure to pesticides (Frixione and Rodríguez-Estrella, 2020) and higher abundance of ectoparasites (Wemer et al., 2021). Urban landscapes can also widely differ in factors like amount of green space, population density and type of buildings, highlighting that general patterns about the impact of cities on birds of prey are not easily predictable (Costantini and Dell'Omo, 2020).

It is increasingly recognised that elucidating the proximate mechanisms that drive the responses of organisms to the ongoing environmental changes has major implications for the adaptability or acclimatisation of animals to the Anthropocene. Several studies suggested that the quantification of gene expression, using high-throughput sequencing (RNA-sequencing), might enable the identification of genes and metabolic pathways that play major roles in the responses of organisms to the changing environments (e.g., Harris et al. 2013, 2015; Watson et al., 2017; Giraudeau et al. 2020; Thompson et al., 2022; Minias 2023). In environmental epidemiology, it is increasingly recognised that omic technologies might enable a better characterisation of the exposome, which includes the lifetime exposure of organisms to internal and external biological, chemical and physical agents (with the exclusion of genetic factors) that can impact on health (Wild 2005; Vineis et al. 2020). However, there are limitations that need to be considered. For example, differences in expression profiles are expected among different tissues (e.g. Watson et al. 2017). Also, protein levels are not proportional to mRNA levels (e.g. Lu et al. 2007; Lundberg et. al. 2010). Thus, inferences about phenotypic or fitness differences between birds living in contrasting

environments need to be substantiated by analyses of specific biomarkers or reproductive traits.

In this study, we have compared for the first time the blood transcriptome of common kestrel nestlings living in an urban area with that of nestlings inhabiting rural and natural environments. Organisms living in urban and non-urban environments face different challenges and opportunities, yet little is known about responses at the molecular level. With this approach, we aim to reveal genes and pathways that might drive proximate responses of birds to different environments. We focussed on nestlings because they are better suited than adults to address our study questions. Although some adult kestrels are year-round residents in our study region (Damiani et al. 2022), we cannot exclude the possibility that some individuals may disperse or migrate from other environments. This would mean that, differently from nestlings, the transcriptome of adults might not entirely reflect the molecular responses to the local environment.

In previous work, we found lower concentrations of glutathione, lower GSH:GSSG ratio values, and higher proportions of monocytes in urban kestrel nestlings compared to both rural and natural kestrel nestlings (Giovanetti et al. 2024). We also found higher DNA damage in rural kestrel nestlings compared to both urban and natural kestrel nestlings, and higher inhibition of butyrylcholinesterase activity in urban and natural kestrel nestlings compared to rural kestrel nestlings (Giovanetti et al. 2024). We also have some indications for differences in reproductive traits between urban and non-urban kestrels (e.g. laying date, *data in preparation*). Thus, transcriptome analysis might also help to infer potential factors that generated this differentiation in certain biomarkers between urban and non-urban kestrels.

Materials and methods

Study area and sample collection

The study area included the city of Rome and two sites in the countryside of the city, which were characterised by a mosaic of land uses and human exploitation (Figure 1). We calculated an urbanisation gradient (UG) as the percentage of built or cemented soil (buildings and traffic) within 500 metres of each sampled nest, following Kübler et al. (2005) and Sumasgutner et al. (2014a) (QGIS v3.10 software). The urban site (URB) included the city centre of Rome, which is characterised by a preponderant matrix of buildings, roads and human structures (UG = 46.94%), and small green areas (urban parks and house gardens). The rural site (RUR) is located in the region of Decima Malafede, about 10 km from Rome. This site is delimited by busy roads (Grande Raccordo Anulare, Pontina, and Laurentina roads) and is mainly characterised by croplands and grasslands (UG = 7.04%). The natural site (NAT) is located in Castel di Guido within the *Litorale Romano* Nature Reserve, 20 km from Rome city centre. This site is mainly characterised by woods (*Quercus* sp.), Mediterranean scrub, set asides, and pasturelands (UG = 2.04%).

We monitored the breeding activity of 45 pairs of kestrels that bred in nest boxes (50 × 30 × 30 cm) on high-voltage power lines (from Terna and Acea) from April to July of 2022. The use of nest boxes enabled us to standardise the breeding conditions to avoid any potential variation in reproduction or physiology owing to the type of breeding site (Lambrechts et al. 2012). Moreover, GPS tracking revealed that kestrels have a small home range, meaning they are mainly influenced by the local conditions surrounding the nesting site (Damiani et al. 2022). We collected a sample of whole blood (~0.4 ml) from the metatarsal vein of nestlings using a 1 ml syringe (Owen, 2011;

Klein and Sojka, 2012). The blood was straightway mixed with a buffer (RNAprotect Animal Blood Tubes, QIAGEN Inc.) to avoid RNA degradation. Then, we stored the tubes at -80°C until the laboratory analyses were conducted. In total, we collected blood samples from six URB nestlings from four nests, four RUR nestlings from four nests, and five NAT nestlings from five nests. All blood samples were taken in the morning over four days in June to avoid any effect of circadian and seasonal variation; both the time and day of blood collection was the same across the three sites ($F = 0.46$, $p = 0.65$ and $F = 0.64$, $p = 0.54$, respectively). Our sample size was modest but in line with other studies on transcriptomics (e.g. 12 in Watson et al. 2017; 9 in Blackburn et al. 2021; 18 in Cheng et al. 2023; 9 in Yang et al. 2023), which usually require moderate samples sizes owing to the large amount of data that can be obtained. Thus, our sample size of 15 nestlings can be considered prudent and suitable to detect only major differences, thereby rendering our results somewhat conservative.

RNA extraction

We performed the purification of total RNA, excluding miRNA (<200 nucleotides), using the RNeasy Protect Animal Blood Kit (Qiagen, Germany). We briefly centrifuged blood samples to pellet them, then washed them with RNase-free water and re-suspended them using a buffer. After digestion in a buffer containing guanidine thiocyanate (RBT) with proteinase K, we homogenised the samples by centrifugation through QIAshredder spin columns. Then we added ethanol to the samples, which were centrifuged again through RNeasy MinElute spin columns. We removed the bound total DNA using a DNase digestion and washed them using two buffers. We then eluted the pure RNA using an elution buffer. Finally, we quantified the amount, integrity and

purity of the RNA using the Agilent Bioanalyzer 5400 system (Agilent Technologies, CA, USA). Next, we shipped the extracted RNA samples to Novogene Co. for sequencing.

Library construction and quality control

Messenger RNA was purified from total RNA using poly-T oligo-attached magnetic beads. After fragmentation, the cDNA strands were synthetised using both directional and non-directional libraries and random hexamer primers (Parkhomchuk et al., 2009). Each library was checked with Qubit and real-time PCR for quantification and bioanalyzer for size distribution detection.

Data were cleaned by removing low-quality reads and reads containing adapter and poly-N. Reference genome and gene model annotation files were downloaded from https://www.ncbi.nlm.nih.gov/datasets/taxonomy/100819/. HISAT2 v.2.0.5 was used to build an index of reference genome and to align paired-end clean 1 reads to the reference genome (Mortazavi et al., 2008). HISAT2 Feature Counts v1.5.0-p3 was used to count the reads mapped to each gene (Liao et al., 2014). The expected number of Fragments Per Kilobase of transcript sequence per Millions base pairs sequenced (FPKM) of each gene was calculated based on the length of the gene and reads count mapped to this gene.

Differential expression analysis

We performed the differential expression analysis using the DESeq2 R package 1.20.0 (Anders and Huber, 2010). We adjusted the p-values using the Benjamini and Hochberg's approach for controlling the false discovery rate (Green and Diggle, 2007).

Differentially expressed genes are those with changes in read counts or expression levels and are mostly responsible for biological differences between studied groups (Anjum et al., 2016). We quantified the differences between the three areas, urban (URB), rural (RUR) and natural (NAT). We did this by relying on the number of differentially expressed genes between them (NAT vs. URB, RUR vs. URB, NAT vs. RUR), and, subsequently, identifying the most differentiated pathways through the enrichment analysis (Chen et al., 2017). We used adjusted p-values < 0.05 to select the differentially expressed genes.

Enrichment analysis

Gene Ontology (GO) enrichment analysis of differentially expressed genes was implemented by the cluster Profiler R package, in which gene length bias was corrected (Young et al., 2010). Significant GO terms (*p-value* < 0.05) were considered enriched by differentially expressed genes. The database KEGG was used for understanding high-level functions and utilities of biological systems. KEGG pathway enrichment was used to identify the most significant pathways that differ the most between different groups (Kanehisa and Goto, 2000; Kanehisa, 2002).

Results

High-throughput sequencing generated 64,619,844 to 104,561,300 total clean reads per individual, mapping between 74.87% and 83.56% of the genome. The percentage of reads being mapped to unique positions of the reference genome (for subsequent quantitative data analysis) ranged between 71.42% and 78.67% per individual, a much higher percentage than the 60% basic requirement for non-model organisms.

Sequencing error rates ranged from 0.02% to 0.03%, and the GC content percentage was above 52% in all individuals.

In the NAT-URB comparison, we identified a total of 58 differentially expressed genes, with 40 upregulated genes and 18 downregulated genes in NAT compared to URB (Figure 2, Table 1). In Table 1, we also reported six additional genes that were marginally different between NAT and URB. In the RUR-URB comparison, we identified 12 differentially expressed genes, with eight upregulated genes and four downregulated genes in RUR compared to URB (Figure 2, Table 1). Finally, in the NAT-RUR comparison, we only identified one differentially expressed gene that was downregulated in NAT, and one gene whose expression was marginally different between the two areas (Figure 2, Table 1).

Relying on log2 fold change values, the most striking differences were found in the comparison between NAT and URB kestrel nestlings for the genes *FLNC* (5.81), *C1QTNF7* (-4.46) and *DDX39B* (3.63), and in the comparison between RUR and URB kestrel nestlings for the genes *CHAC1* (4.46) and *ACOX3* (3.19) (Table 1).

The GO enrichment analysis did not identify any differences in cellular components, biological processes, or molecular functions among sites. The KEGG enrichment analysis identified a marginally significant difference between NAT and URB in the insulin signalling pathway (p-adj = 0.055), and three other pathways with a p-adj less than 0.1: carbon metabolism, p-adj = 0.070; pyruvate metabolism, p-adj = 0.085; and apoptosis, p-adj = 0.085 (Figure 3).

Discussion

The results of our study show small differences in blood transcriptome among common kestrel nestlings growing in environments with different intensities of human disturbance. We found stronger differences in gene expression between NAT and URB kestrel nestlings and, to a smaller degree, between RUR and URB kestrel nestlings. By contrast, the gene expression profile was very similar between NAT and RUR kestrel nestlings. Our results were robust for variation in both time and season of blood collection, and in breeding site (all were in nest-boxes). However, our modest sample sizes could have limited our power to detect more differences in the expression of genes, making our results somewhat conservative and limited only to those genes that showed strong differences among sites.

As compared to common kestrel nestlings from the natural area, we found that urban nestlings showed higher expression of several genes that regulate inflammatory or acquired immune responses, such as the complement C1q tumour necrosis factor-related protein 7 (*C1QTNF7* gene), the zinc finger C (*ZXDC* gene) that cooperates with other genes to promote transcription of major histocompatibility complex classes I and II genes, and the complement C3b/C4b receptor 1 (*CR1* gene) that contributes to the capture and clearance of complement-opsonised pathogens. The higher expression of inflammatory genes is in line with a prior study carried out in the same study sites that found higher proportions of monocytes (type of innate immune cells involved in the inflammatory response) in URB than NAT kestrel nestlings (Giovanetti et al. 2024). We also detected differences between RUR and URB nestlings in some genes with a link to immune function. Compared to URB nestlings, RUR nestlings had upregulated expression of the (i) *MYLK3* gene, which has among its related pathways the immune response CCR3 signalling in eosinophils, and (ii) *UBE2Q2* gene, which has among its

related pathways the Class I major histocompatibility complex mediated antigen processing and presentation. These results are partly in agreement with previous studies on urban mice, blue tits, and great tits that found upregulation of immunological genes in urban populations compared to rural populations (Harris et al. 2015; Capilla-Lasheras et al. 2017; Watson et al. 2017). It could be that urban populations are being exposed to higher levels of chemical pollutants that cause tissue inflammation (Jalaludin, 2014; Isaksson, 2015). However, we also found that NAT nestlings had higher expression of some genes linked to the development of B immune cells, T cell activation, and promotion of the type I interferon response (e.g. *BLNK*, *CD82*, *DDX39B*,). In particular, we found that the genes *BLNK* and *CD82* were upregulated in NAT nestlings compared to URB nestlings, while a previous study on great tits in Sweden found opposite results for both genes (Watson et al. 2017). These results might suggest that diversity and abundance of parasites or pathogens and likelihood of infection vary significantly between urban and non-urban habitats depending on the species and the landscape structure (e.g. McKinney, 2008; Evans et al., 2009; Murray et al. 2019; Wemer et al., 2021; Perrin et al., 2022). For example, prior work on kestrels in Wien found greater abundance of ectoparasites in rural than urban populations (Wemer et al., 2021). Similarly, studies on other bird species or mammals also found that some parasites or pathogens can become rare or disappear in urban populations (e.g. Gregoire et al. 2002; Mdegela et al. 2006; Hegglin et al. 2007). This hypothesis on variation in pathogen or parasite pressure between urban and non-urban environments is also supported by studies that found different polymorphisms linked to major histocompatibility complex genes (which code for cell surface proteins essential for the adaptive immune system)

between conspecific vertebrates living in environments differing in urbanisation level (e.g. DeCandia et al. 2019a,b; Pikus et al. 2021).

Urban nestlings also showed upregulation of some genes that are involved in the repair of DNA damage and cell apoptosis, such as the *WDR4*, *APTX*, and *UHRF1*. The relevance of the apoptosis pathway was further corroborated by the KEGG enrichment analysis. Urban nestlings might be exposed to genotoxic stressors that require higher activity of the DNA repair systems. One reason for this difference might lie with the exposure to pollutants. In our study region, urban kestrels are exposed to higher levels of some types of organic pollutants (e.g. DDE, PCBs, HCB) compared to kestrels living outside the urban environment (Dell'Omo et al. 2008). Other studies on birds also found more active repair systems in urban than non-urban populations (Watson et al. 2017). It might be that upregulation of DNA repair genes is needed to limit mutations and prevent cancerogenesis (Sepp et al. 2019; Giraudeau et al. 2020). Compared to RUR, urban kestrels also showed upregulation of the oncogene Subgroup A Rous sarcoma virus receptor pg950. However, NAT nestlings had one gene linked to tumour suppression (*ZNF292*) and RUR nestlings had two genes linked to apoptosis (*CHAC1* and *SLC25A6*) that were significantly upregulated compared to URB kestrels. Previous work in the same study region found that levels of DNA damage (comet assay) were higher in RUR than in URB or NAT nestlings, suggesting that upregulation of DNA repair genes might play a non-negligible role in preventing accumulation of damage (Giovanetti et al. 2024). Further studies are needed to identify potential genotoxic agents, to quantify how these agents vary across different environments, and to elucidate their effects on the expression of anti-cancer defences.

The comparison between NAT and URB nestlings further identified several differentially expressed genes that regulate development (*FLNC*), lipid metabolism (*ST8SIA1*) and insulin activity (*INPPL1*). Despite previous studies on other bird species suggested that the rate and pattern of growth of urban nestlings can differ from those of non-urban nestlings (e.g. Corsini et al. 2021), we do not know if development genes are differentially expressed because the growth rate of kestrels differed between urban and non-urban sites. Our present results show that these differences in development genes were associated with those of genes regulating nutrient pathways. In particular, the KEGG enrichment analysis identified insulin signalling as the main pathway that differed between NAT and URB nestlings. A previous study on the bananaquit (*Coereba flaveola*) suggested that insulin pathway might be under selection in urban environments (Mascarenhas et al. 2023). Carbon and pyruvate metabolism were also relevant pathways, further pointing to a metabolic differentiation between NAT and URB nestlings. Although to a lower extent, we also found evidence for a differential metabolic regulation between RUR and URB nestlings. In particular, RUR nestlings showed higher expression of the *ACOX3* gene, which regulates peroxisomal lipid metabolism and fatty acid metabolism. Sites did not differ in time or season of bleeding, allowing us to exclude any potential noise owing to circadian or seasonal variation in the expression of metabolic genes. However, since we do not know if all nestlings were recently fed by parents, we cannot control for any potential effect owing to digestion. Irrespective of these factors, our results are in line with studies on other bird species. Prior work on adult great tits also found that several genes that were significantly differentially expressed between the urban and rural birds were involved in the regulation of metabolism and storage of fatty acids (Watson et al. 2017). Similarly,

genomic analyses of adult white-footed mice found that many genes that were associated with variables related to urbanisation had roles in the metabolism of lipids and carbohydrates (Harris and South 2017).

Experimental studies on laboratory models showed that exposure to particulate matter with a diameter less than 2.5 μm can cause insulin signalling inhibition (Zhang et al. 2021). Metabolism also exhibits plasticity in response to variation in quality, availability, and predictability of food (*food habits hypothesis*; e.g. McNab 1986; Norin and Metcalfe 2019; Auer et al., 2020). We do not have estimates of diet composition across different environments. However, previous work on populations of common kestrels in northern regions of Europe found a higher percentage of passerine birds in the diet of urban kestrels (e.g. Kübler et al., 2005; Kreiderits et al., 2016). In northern regions, kestrels primarily feed on voles. It has been hypothesised that this trophic shift could be responsible for the poor reproductive performance of kestrels in urban habitats (Sumasgutner et al., 2014b). However, in the Mediterranean region, kestrels have a wider trophic niche, and voles do not represent the main prey (Costantini et al. 2005). This topic deserves further investigation into the variation in growth rates, diet and exposure to endocrine disruptors among sites.

The results of our study are limited to a single tissue, thus replication in other tissues is needed to better characterise the organismal transcriptomic response to the prevailing environmental conditions (e.g. Watson et al., 2017). Although some of the environmentally-induced functional responses might operate in more than one tissue, they might involve different genes (e.g. Watson et al., 2017). The choice of the tissue also depends on the specific questions to be addressed and ethical considerations. For example, liver might be relevant for studies on nutritional and metabolic pathways,

while specific areas of the brain would be more appropriate for studies on how the environment impacts on neurogenesis or cognition. We have chosen the blood because previous work on kestrel nestlings found differences between sites in blood levels of DNA damage, glutathione and monocytes (Giovanetti et al. 2024).

Conclusions

Our study provides the first investigation of the whole transcriptome in a bird of prey across urban and non-urban environments. The most striking differences among sites involved inflammatory-immunological, metabolic, apoptosis, DNA repair and development genes. These results suggest that environmental pressures might differ in typology and intensity across habitats requiring specific endogenous responses, which is supported by a recent multi-biomarker study carried out on kestrel nestlings in the same study region (Giovanetti et al. 2024). We do not know if these differences in gene expression are relevant for fitness. We suggest that future studies on common kestrels or other bird species living in contrasting environments also focus on the reproductive ecology and survival to determine the ecological relevance of different patterns of gene expression.

Acknowledgments

We thank Terna s.p.a. and ACEA s.p.a. for granting access to the power lines and the nest boxes. We also thank Michela Paoletti and Andrea Chiocchio for advice on RNA extraction. We thank Megan O'Neil for revising the language. We thank the two reviewers and the editor for providing constructive criticisms that helped us improve the manuscript. The work was authorised by the Institute for Environmental Protection and

Research ISPRA (protocol n. 12495/2020 and 11640/2022) and the Regione Lazio (protocol n. G03417/2020 and G04115/2022).

Funding

This research was funded by *Ornis italica* (Italy) and the CNRS (France). G. Damiani was supported by a PhD scholarship D.M. 117/2023 funded by 'NextGenerationEU, Piano Nazionale di Ripresa e Resilienza (PNRR) – MUR'.

Data availability

Data will be made available on request.

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Figure captions

Figure 1. The image illustrates the three study areas (white dashed lines) in and around the city of Rome: URB = urban; RUR = rural; NAT = natural. White points indicate the locations of the nestlings. Light yellow lines indicate the main roads crossing the study area.

Figure 2. Volcano plots of differentially expressed genes between natural (NAT) and urban (URB) common kestrels (top panel), rural (RUR) and URB kestrels (middle panel), and NAT and RUR kestrels (bottom panel). The black dots represent the genes that do not differ between groups, and the orange and light-blue dots represent the differentially downregulated and upregulated genes, respectively.

Figure 3. KEGG enrichment analyses of differential expressed genes between urban and natural common kestrels. The size of each point is related to the number of genes corresponding to that term, according to the figure legend. The Gene ratio refers to the ratio of differentially expressed genes over all genes of a specific KEGG pathway. The adjusted p-value decreases from top to bottom.

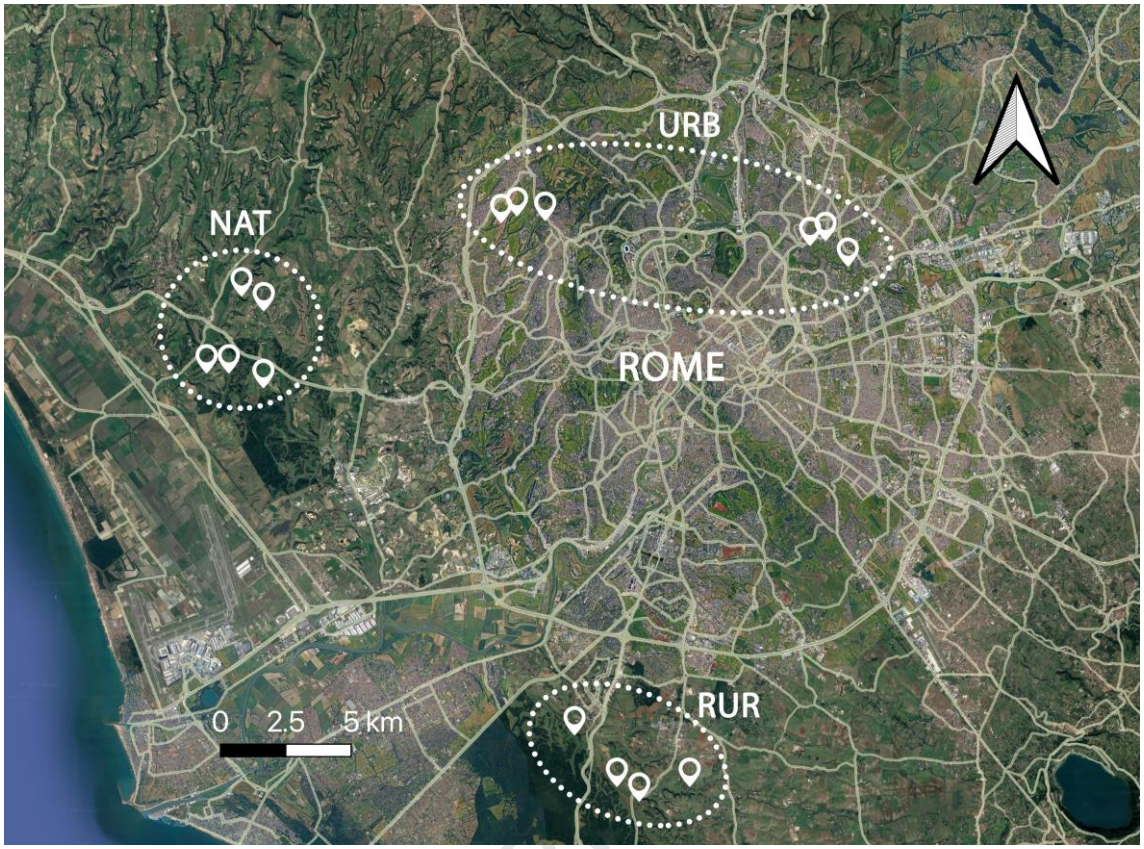


Fig. 1

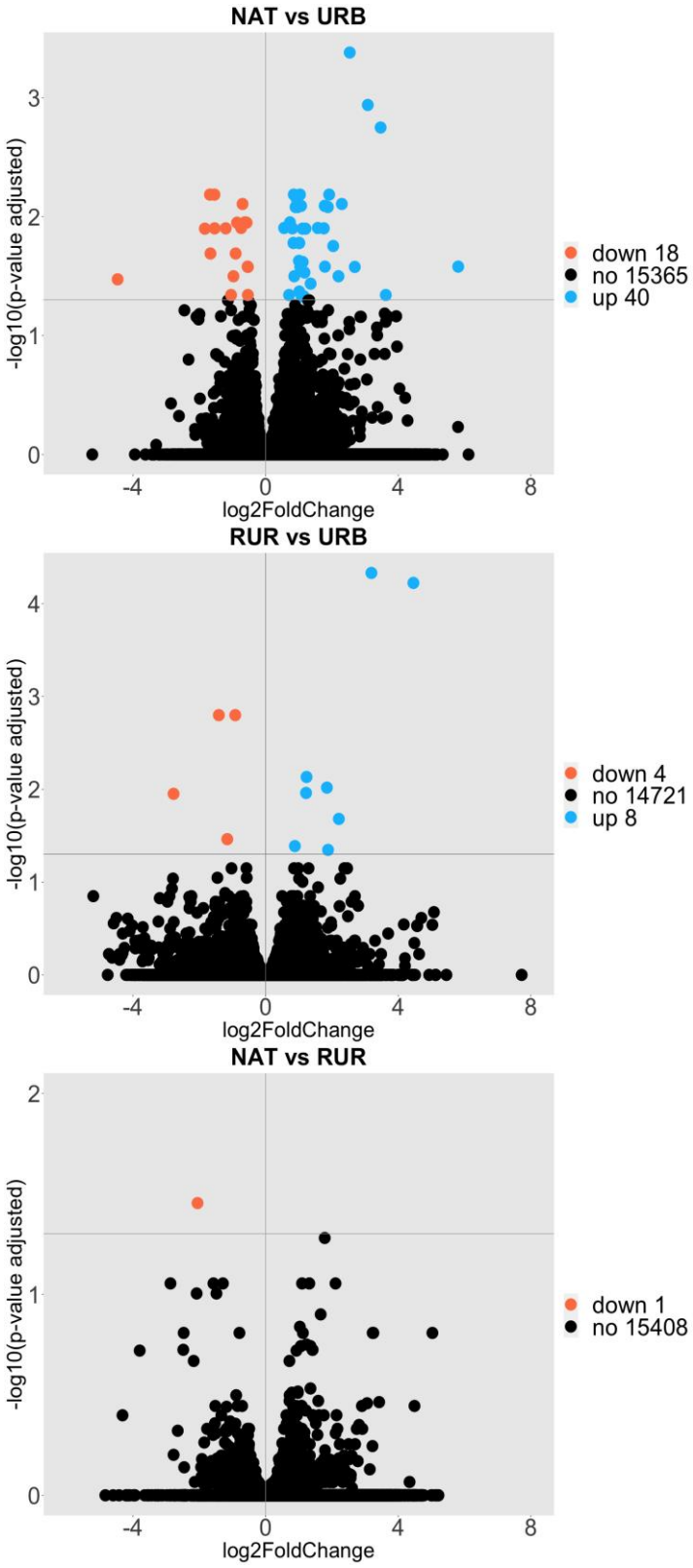

NAT vs URB
-log10(p-value adjusted)
log2FoldChange
down 18
no 15365
up 40
RUR vs URB
-log10(p-value adjusted)
log2FoldChange
down 4
no 14721
up 8
NAT vs RUR
-log10(p-value adjusted)
log2FoldChange
down 1
no 15408

Fig. 2

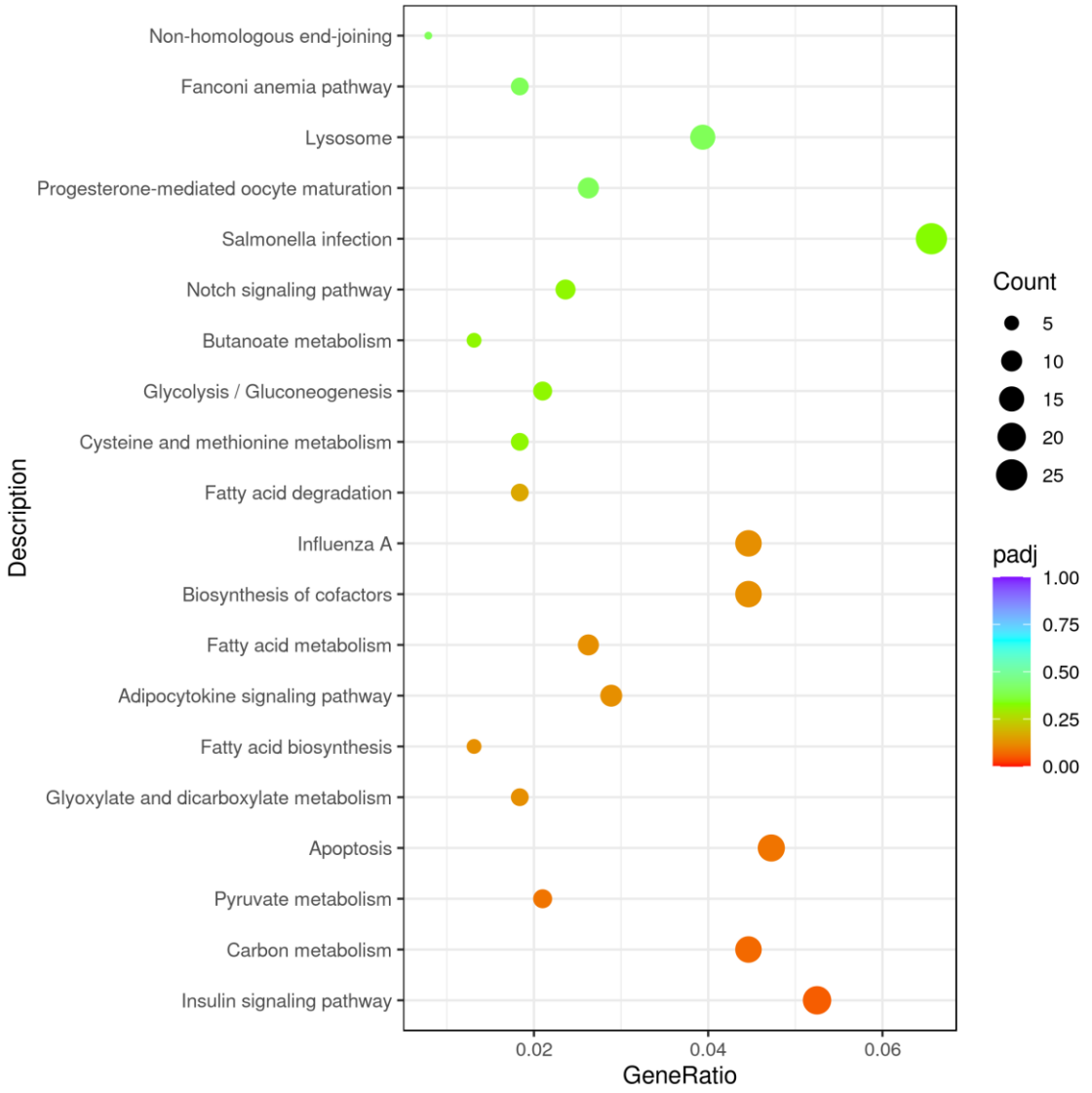


Fig. 3

Table 1. List of differentially expressed genes and their functions. Adjusted p-values that are not shown in italics refer to those genes that were marginally different between groups. Information about the functions of genes was obtained from https://www.genecards.org/, https://www.proteinatlas.org/, https://david.ncifcrf.gov/home.jsp, https://www.ncbi.nlm.nih.gov/ and https://string-db.org/cgi/input?sessionId=bNUIk3P999w4&input_page_show_search=on. Log2FC = log2 fold change.

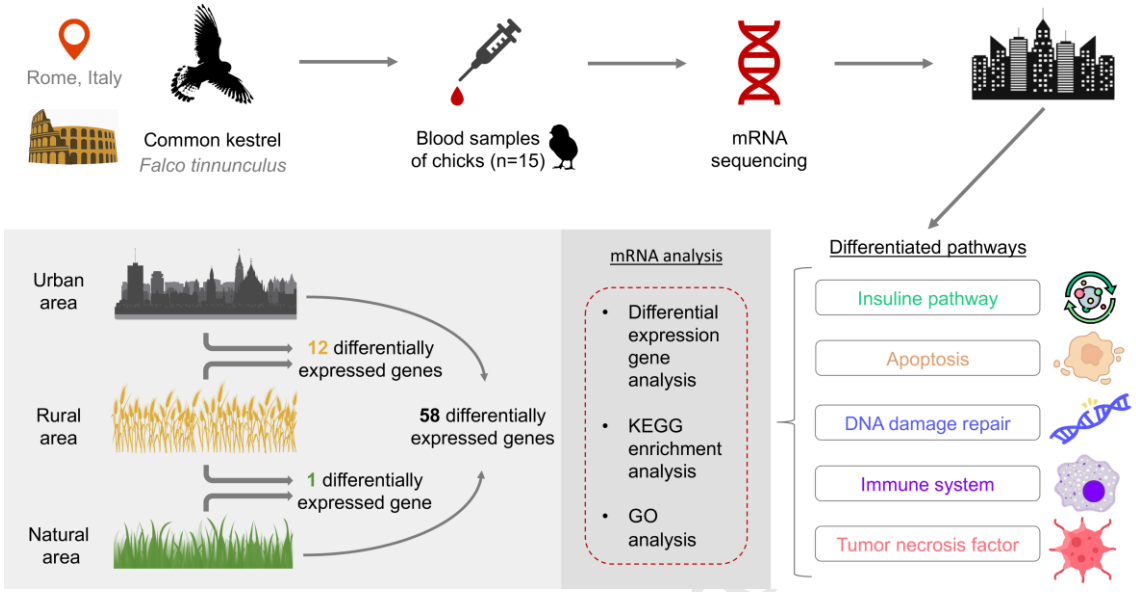
Gene ID	**Higher expression in**	**log2FC**	**adj-P**	**Gene name**	**Function**
NAT vs URB					
ENSFTIG00000013700	natural	2.54	*0.00042*	unidentified	
ENSFTIG00000001778	natural	3.08	*0.00115*	C6orf132 Gene - Chromosome 6 Open Reading Frame 132	Uncharacterized protein.

ENSFTIG00000001480	natural	3.47	*0.00178*	UNC80 Gene - Unc-80 Homolog	Component of a voltage-independent leak ion-channel complex, growth.
ENSFTIG00000000665	natural	1.92	*0.00652*	CLUS Gene - Clusterin	Cell death, tumor progression, immune response, and neurodegenerative disorders.
ENSFTIG00000000817	natural	0.85	*0.00652*	CTSZ Gene - Cathepsin Z	General protein turnover, antigen processing and bone remodelling.
ENSFTIG00000005423	natural	1.04	*0.00652*	EVI5L Gene - Ecotropic Viral Integration Site 5 Like	Cell cycle progression, cytokinesis, and cellular membrane traffic.
ENSFTIG00000014568	urban	-1.68	*0.00652*	CFAP96 Gene - Cilia And Flagella Associated Protein 96	Cell structure.
ENSFTIG00000015417	urban	-1.54	*0.00652*	TBX4 Gene - T-Box Transcription Factor 4	Limb development.
ENSFTIG00000004884	natural	0.94	*0.00706*	NT5C3A Gene - 5'-Nucleotidase, Cytosolic IIIA	Hydrolysis of nucleotides, immunity.
ENSFTIG00000002607	urban	-0.70	*0.00782*	TRUB2 Gene - TruB Pseudouridine Synthase Family Member 2	Mitochondrial function.
ENSFTIG00000012342	natural	0.98	*0.00782*	LIMK1 Gene - LIM Domain Kinase 1	Development, cytoskeletal structure.
ENSFTIG00000001187	natural	2.30	*0.00782*	TMEM37 Gene - Transmembrane Protein 37	Calcium ion transmembrane transport.
ENSFTIG00000002183	natural	1.78	*0.00810*	VAT1 Gene - Vesicle Amine Transport 1	Epidermal repair mechanisms, oxidoreductase.
ENSFTIG00000015743	natural	1.07	*0.00810*	CD82 Gene - CD82 Molecule	T cell activation, metastasis suppressor.
ENSFTIG00000006999	natural	0.96	*0.00829*	RYK Gene - Receptor Like Tyrosine Kinase	Transfer a phosphate group from ATP to the tyrosine residues.
ENSFTIG00000004529	natural	1.88	*0.00829*	WDFY3 Gene - WD Repeat And FYVE Domain Containing 3	Autophagy of aggregated proteins, development.
ENSFTIG00000015775	natural	0.90	*0.00829*	MFSD2B Gene - MFSD2 Lysolipid Transporter B, Sphingolipid	Lipid transport required for red blood cell morphology, platelet aggregation, and thrombus formation.
ENSFTIG00000004093	urban	-0.63	*0.01116*	PPM1G Gene - Protein Phosphatase, Mg2+/Mn2+ Dependent 1G	Apoptosis, negative regulator of cell stress response.
ENSFTIG00000003688	natural	0.73	*0.01116*	PPOX Gene - Protoporphyrinogen Oxidase	Heme biosynthesis, generation of protoporphyrin IX.
ENSFTIG00000010698	urban	-0.57	*0.01122*	WDR4 Gene - WD Repeat Domain 4	Cell cycle progression, signal transduction, apoptosis, and gene regulation; regulation of DNA damage repair.
ENSFTIG00000013911	urban	-0.86	*0.01122*	APTX Gene - Aprataxin	Single-strand and double-strand DNA break repair, base excision repair.
ENSFTIG00000000161	natural	1.57	*0.01244*	SHMT2 Gene - Serine Hydroxymethyltransferase 2	Mitochondrial function and ROS generation; SHMT2 knockdown triggers cell apoptosis.
ENSFTIG00000000209	natural	0.56	*0.01244*	HDAC1 Gene - Histone Deacetylase 1	Control of cell proliferation and differentiation.
ENSFTIG00000007940	urban	-0.74	*0.01244*	CR1 Gene - Complement C3b/C4b Receptor 1 (Knops Blood Group)	Capture and clearance of complement-opsonized pathogens.
ENSFTIG00000007587	natural	1.76	*0.01253*	ST8SIA1 Gene - ST8 Alpha-N-Acetyl-Neuraminide Alpha-2,8-Sialyltransferase 1	Lipid metabolism.
ENSFTIG00000012999	urban	-1.54	*0.01253*	ESRRB Gene - Estrogen Related Receptor Beta	Development.
ENSFTIG00000014869	natural	0.81	*0.01253*	TMOD3 Gene - Tropomodulin 3	Development.
ENSFTIG00000003737	urban	-1.20	*0.01253*	ERMARD Gene - ER Membrane Associated RNA Degradation	Development.
ENSFTIG00000001306	natural	1.20	*0.01261*	NMD3 Gene - NMD3 Ribosome Export Adaptor	Involved in the transport of ribosomes.

ENSFTIG00000003186	natural	1.11	*0.01261*	ABCC4 Gene - ATP Binding Cassette Subfamily C Member 4	ATP-dependent transporter of macromolecules and xenobiotics from cells.
ENSFTIG00000011246	urban	-1.83	*0.01261*	SNX24 Gene - Sorting Nexin 24	Protein transport.
ENSFTIG00000003762	natural	0.84	*0.01663*	ZYX Gene - Zyxin	Promotion of type I interferon response, potentially associated with increased resistance to experimental avian coccidiosis. Zyxin takes part in apoptosis, as well as in wound healing.
ENSFTIG00000000007	natural	1.02	*0.01663*	ZNF292 Gene - Zinc Finger Protein 292	Involved in tumor suppression.
ENSFTIG00000010665	natural	1.01	*0.01663*	LPAR6 Gene - Lysophosphatidic Acid Receptor 6	Development.
ENSFTIG00000001783	natural	2.04	*0.01764*	SLIT1 Gene - Slit Guidance Ligand 1	Development.
ENSFTIG00000003694	urban	-1.66	*0.02040*	PFN4 Gene - Profilin Family Member 4	Development.
ENSFTIG00000001915	urban	-0.90	*0.02040*	NUP155 Gene - Nucleoporin 155	mRNA and protein transport.
ENSFTIG00000011010	natural	1.00	*0.02352*	INPPL1 Gene - Inositol Polyphosphate Phosphatase Like 1	Regulation of insulin function.
ENSFTIG00000006006	natural	1.12	*0.02405*	PPP1R26 Gene - Protein Phosphatase 1 Regulatory Subunit 26	Inhibition of phosphatase activity.
ENSFTIG00000002568	urban	-0.54	*0.02630*	KNTC1 Gene - Kinetochore Associated 1	Chromosome segregation during cell division.
ENSFTIG00000000733	natural	5.81	*0.02630*	FLNC Gene - Filamin C	Development, particularly of muscle tissue.
ENSFTIG00000001318	natural	1.79	*0.02630*	CAPN5 Gene - Calpain 5	Calcium-dependent cysteine proteases involved in signal transduction.
ENSFTIG00000015121	urban	-0.53	*0.02644*	TM9SF2 Gene - Transmembrane 9 Superfamily Member 2	Small molecule transport, ion channel.
ENSFTIG00000016009	natural	1.04	*0.02644*	PTP4A3 Gene - Protein Tyrosine Phosphatase 4A3	Cell proliferation.
ENSFTIG00000002712	natural	2.69	*0.02644*	F8A1 Gene - Coagulation Factor VIII Associated 1	Vescicular trafficking.
ENSFTIG00000000481	natural	1.17	*0.02946*	RWDD2A Gene - RWD Domain Containing 2A	Protein degradation, ubiquitination.
ENSFTIG00000002324	urban	-0.97	*0.03168*	UHRF1 Gene - Ubiquitin Like with PHD And Ring Finger Domains 1	DNA-binding transcription factor activity and ligase activity; linked to p53-dependent DNA damage checkpoint.
ENSFTIG00000000362	natural	2.20	*0.03168*	THBD Gene - Thrombomodulin	Endothelial-specific type I membrane receptor that binds thrombin.
ENSFTIG00000009372	natural	0.86	*0.03168*	KMT5A Gene - Lysine Methyltransferase 5A	Cell proliferation and chromatin condensation.
ENSFTIG00000006227	urban	-4.46	*0.03366*	C1QTNF7 Gene - Complement C1q Tumor Necrosis Factor-Related Protein 7	Inflammation.
ENSFTIG00000014753	natural	1.36	*0.03655*	SLC6A9 Gene - Solute Carrier Family 6 Member 9	Neurotransmitter:sodium symporter activity and amino acid:sodium symporter activity.
ENSFTIG00000007582	natural	1.02	*0.04261*	MAPK7 Gene - Mitogen-Activated Protein Kinase 7	Proliferation, differentiation, transcription regulation and development.
ENSFTIG00000007220	natural	3.63	*0.04556*	DDX39B Gene - DExD-Box Helicase 39B	mRNA transport, associated with immune genes.
ENSFTIG00000009157	urban	-0.53	*0.04556*	ZXDC Gene - ZXD Family Zinc Finger C	Promote transcription of MHC class I and MHC class II genes.
ENSFTIG00000003645	natural	0.71	*0.04556*	DTX4 Gene - Deltex E3 Ubiquitin Ligase 4	Involved in Notch signaling pathway and protein ubiquitination.
ENSFTIG00000008528	urban	-1.04	*0.04556*	SUSD1 Gene - Sushi	Calcium ion binding, highly

				Domain Containing 1	expressed in immune cells.
ENSFTIG00000009571	natural	1.15	*0.04672*	BMI1 Gene - BMI1 Proto-Oncogene, Polycomb Ring Finger	Epigenetic repressor of multiple regulatory genes involved in embryonic development and self-renewal in somatic stem cells; DNA damage repair.
ENSFTIG00000006187	natural	1.09	*0.04672*	BLNK Gene - B Cell Linker	B cell development.
ENSFTIG00000006412	urban	-1.14	0.05058	CCDC171 Gene - Coiled-Coil Domain Containing 171	DNA-binding transcription factor activity
ENSFTIG00000008684	natural	1.32	0.05058	TSPO Gene - Translocator Protein	Flow of cholesterol into mitochondria.
ENSFTIG00000002937	natural	1.28	0.05068		
ENSFTIG00000007839	urban	-0.50	0.05174	TYW1 Gene - TRNA-YW Synthesizing Protein 1 Homolog	Probable component of the wybutosine biosynthesis pathway.
ENSFTIG00000008468	urban	-0.44	0.05559	SEC31A Gene - SEC31 Homolog A, COPII Coat Complex Component	Formation of transport vesicles from the endoplasmic reticulum.
ENSFTIG00000011975	natural	0.89	0.05559	TP53 Gene - Tumor Protein P53	Response to diverse cellular stressors, inducing cell cycle arrest, apoptosis, senescence, DNA repair, or changes in metabolism.
RUR vs. URB					
ENSFTIG00000003423	rural	3.19	*0.000047*	ACOX3 Gene - Acyl-CoA Oxidase 3, Pristanoyl	Peroxisomal lipid metabolism and fatty acid metabolism.
ENSFTIG00000009358	rural	4.46	*0.000060*	CHAC1 Gene - ChaC Glutathione Specific Gamma-Glutamylcyclotransferase 1	Regulation of glutathione levels and oxidative status, inhibition Notch signalling, pro-apoptotic component of the unfolded protein response pathway.
ENSFTIG00000006253	urban	-1.41	*0.001590*	PHLDB1 Gene - Pleckstrin Homology Like Domain Family B Member 1	Regulation of microtubule cytoskeleton organization.
ENSFTIG00000001113	urban	-0.92	*0.001590*	BAHCC1 Gene - BAH Domain and Coiled-Coil Containing 1	Chromatin binding.
ENSFTIG00000007809	rural	1.23	*0.007373*	UBE2Q2 Gene - Ubiquitin Conjugating Enzyme E2 Q2	Ubiquitin-protein transferase activity. Relation with Class I MHC mediated antigen processing and presentation and metabolism of proteins.
ENSFTIG00000001071	rural	1.85	*0.009605*	MTG2 Gene - Mitochondrial Ribosome Associated GTPase 2	Protein synthesis, nuclear transport, membrane trafficking, and signal transduction.
ENSFTIG00000015537	rural	1.22	*0.010956*	CLIP4 Gene - CAP-Gly Domain Containing Linker Protein Family Member 4	Cytoplasmic microtubule organization.
ENSFTIG00000008785	urban	-2.78	*0.011201*	FSDH_RALPI Phenylserine dehydratase	
ENSFTIG00000007204	rural	2.21	*0.020862*	MYLK3 Gene - Myosin Light Chain Kinase 3	Link with immune response CCR3 signalling in eosinophils.
ENSFTIG00000012818	urban	-1.16	*0.034485*	RSVR_COTJA Subgroup A Rous sarcoma virus receptor pg950	Oncogene.
ENSFTIG00000014916	rural	0.88	*0.040931*	SLC25A6 Gene - Solute Carrier Family 25 Member 6	Translocation of ADP and ATP; apoptosis; mitochondrial uncoupling.
ENSFTIG00000006164	rural	1.88	*0.044990*	AK3 Gene - Adenylate Kinase 3	GTP:ATP phosphotransferase in the mitochondrial matrix.
NAT vs. RUR					
ENSFTIG00000005321	rural	-2.05	*0.035160*	TARBP1 Gene - TAR (HIV-1) RNA Binding Protein 1	RNA binding and RNA methyltransferase activity.
ENSFTIG00000006561	natural	1.78	0.052454	GAB3 Gene - GRB2 Associated Binding Protein 3	Macrophage differentiation.

Graphical abstract



Highlights

- Avian populations are exposed to novel conditions in urban environments.
- Transcriptome analysis revealed differences among urban, natural and rural common kestrel nestlings.
- The main differences involved inflammatory-immunological, metabolic, apoptosis, DNA repair, and development genes.
- Insulin signaling was the main pathway that differed between natural and urban kestrel nestlings.

Declaration of interests

☒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.

☐The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: