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Physiological stress does not increase with urbanization in European blackbirds: Evidence from hormonal, immunological and cellular indicators

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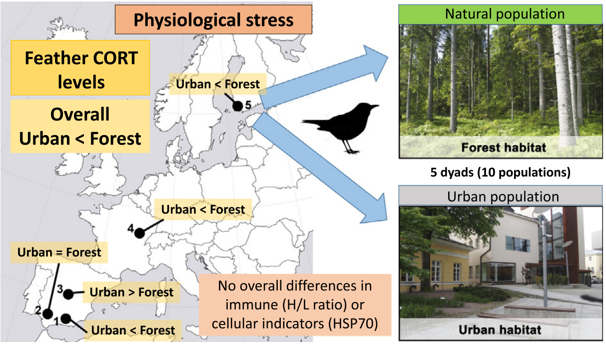
HIGHLIGHTS

- Urban blackbirds do not show higher levels of physiological stress than forest ones.
- Urban areas affect hormonal, immune and cellular indicators differently.
- The effect of urbanization on blackbirds' physiology differs among locations.
- Large scale and multilevel approaches are crucial to study urban effects.

GRAPHICAL ABSTRACT



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ABSTRACT

Urbanization changes the landscape structure and ecological processes of natural habitats. While urban areas expose animal communities to novel challenges, they may also provide more stable environments in which environmental fluctuations are buffered. Species´ ecology and physiology may determine their capacity to cope with the city life. However, the physiological mechanisms underlying organismal responses to urbanization, and whether different physiological systems are equally affected by urban environments remain poorly understood. This severely limits our capacity to predict the impact of anthropogenic habitats on wild populations. In this study, we measured indicators of physiological stress at the endocrine, immune and cellular level (feather corticosterone levels, heterophil to lymphocyte ratio, and heat-shock proteins) in urban and non-urban European blackbirds (*Turdus merula*) across 10 European populations. Among the three variables, we found consistent differences in feather corticosterone, which was higher in non-urban habitats. This effect seems to be

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dependent on sex, being greater in males. In contrast, we found no significant differences between urban and non-urban habitats in the two other physiological indicators. The discrepancy between these different measurements of physiological stress highlights the importance of including multiple physiological variables to understand the impact of urbanization on species' physiology. Overall, our findings suggest that adult European blackbirds living in urban and non-urban habitats do not differ in terms of physiological stress at an organismal level. Furthermore, we found large differences among populations on the strength and direction of the urbanization effect, which illustrates the relevance of spatial replication when investigating urban-induced physiological responses.

1. Introduction

Urbanization dramatically changes the landscape structure and ecological processes of natural habitats. Animals respond to these changes in different ways; illustrated by differences in behavior, morphology or physiology between urban and non-urban populations in a large range of species (Bonier, 2012; Evans, 2010; Sol et al., 2013). While urban areas expose individuals to novel challenges (e.g., resource availability, micro-climate, species interactions or pollution), they may also provide more stable environments in which environmental fluctuations are buffered (Grimm et al., 2008; Schlesinger et al., 2008; Shochat et al., 2006). Organisms differ in their ecological requirements and in the degree of ecological specialisation that has been selected for in the environments in which they have evolved. As a result, environmental modifications deriving from urbanization may have divergent consequences on animals (Grimm et al., 2008; Marzluff et al., 2001), with some species benefiting from the urban environment ("urban exploiters") while others seem unable to survive in cities ("urban avoiders"), or live in the urban environment without relying exclusively on it ("urban adapters"; Blair, 1996).

While the impact of urbanization on animal communities has been well documented (e.g. Hamer and Mcdonnell, 2010; Ibáñez-Álamo et al., 2017; McKinney, 2008), the physiological mechanisms underlying organismal responses to urbanization remain poorly understood, as does whether those mechanisms differ among species (reviewed in Bonier, 2012; Sol et al., 2013). The observed responses are an integration of multiple physiological systems working together, and which may not be equally affected by environmental fluctuations. Identifying the effects of urban habitats on those physiological systems separately, and how they interact at an organismal level is therefore fundamental in order to understand the extent to which species can cope with these anthropogenic habitats, as well as to help design future conservation plans. However, most of the studies investigating physiological responses to urban environments consider only one single variable as indicator of physiological stress (e.g. Abolins-Abols et al., 2016; Partecke et al., 2006). We therefore propose the use of multiple physiological traits as a way to obtain an integrative and more reliable measure of physiological stress of urban populations.

The endocrine system is a primary candidate for mediating organismal responses to environmental challenge (Romero and Wingfield, 2016). Therefore, most of the urban ecology literature has focused on glucocorticoid hormones (e.g. corticosterone –CORT–) to compare exposure to physiological stress of urban and non-urban populations (Bonier, 2012). Glucocorticoid concentrations in blood fluctuate dynamically with energetic needs, increasing in response to perceived (e.g. "stress response") or anticipated environmental changes and integrating physiological responses and associated behaviours through their multiple downstream effects (Buwalda et al., 2012; Jimeno et al., 2018a; McEwen and Wingfield, 2003). CORT concentrations in keratinized tissues, such as feathers ($CORT_f$), are expected to reflect these fluctuations because CORT is deposited continuously from the blood into the tissue as it grows (Jenni-Eiermann et al., 2015). In contrast with plasma CORT, which can be used to infer changes in hormone concentrations over short-time periods, $CORT_f$ reflects changes in circulating CORT (i.e. stress response variation) during the time of feather growth (i.e. weeks), providing an integrated measure of physiological stress over this period (Beaugeard et al., 2019; Fairhurst et al., 2013; Romero and Fairhurst, 2016).

Exposure to urban-associated environmental challenges may also impact immune function (e.g. Audet et al., 2016; Bailly et al., 2016; Capilla-Lasheras et al., 2017; Chávez-Zichinelli et al., 2013). Assessing the differences in immune status between urban vs. non-urban populations is a relevant tool to evaluate potential detrimental consequences of the city life. In birds, heterophils (H) and lymphocytes (L) are the two most abundant white cell types (Davis et al., 2008), and their circulating levels (H/L– ratio) have been widely used to assess immune status in a variety of organisms (e.g. Bókony et al., 2012; Fokidis et al., 2008; French et al., 2008; Ibáñez-Álamo et al., 2016). The H/L ratio is expected to increase under immune challenges from parasites, infection, inclement weather or social competition (Davis et al., 2008; Krams et al., 2011; Minias et al., 2018). However, changes in leukocyte numbers take longer to initiate (i.e. hours or even days) and last longer than changes in other biomarkers such as glucocorticoid levels (Davis et al., 2008; reviewed in O'Dell et al., 2014), potentially offering complementary information on the nature and timing of the physiological challenges faced by the organism.

At the cellular level, one of the main responses to physiological challenges is mediated by heat-shock proteins (HSPs). This family of proteins protect cells from degradation and damage by environmentally-induced instability (Feder and Hofmann, 1999). HSP expression has been found to increase when cells are exposed to challenges such as parasites (del Cerro et al., 2010; Martínez-Padilla et al., 2004; Merino et al., 1998), limited food availability (Herring et al., 2011), predation risk (Thomson et al., 2010) or sibling competition (Martínez-Padilla et al., 2004). Therefore, these molecules are considered reliable indicators of long-lasting challenge (Herring et al., 2011; Martínez-Padilla et al., 2004) and are an excellent complement to hormonal and immune biomarkers.

Overall, the urban ecology literature offers contrasting results regarding the associations between indicators of physiological stress and urbanization. Whereas many studies anticipate a positive association between CORT levels and urbanization assuming that cities represent detrimental environments for animals, this association is not the predominant finding (reviewed in Bonier, 2012), and may only hold true if cities ubiquitously represent energetically challenging environments. Similar predictions have been made for the immune system, as elevated H/L ratios have been previously related to urbanization (H. B. Fokidis et al., 2008) or environmental challenges associated with urban areas such as chemical pollution (Eeva et al., 2005) or habitat fragmentation (Hinam and Clair, 2008). However, these results are not always consistent across studies or species (Bókony et al., 2012; H. B. Fokidis et al., 2008; Ruiz et al., 2002). As for the HSPs, their potential association with urbanization remain unexplored even though many of the factors known to influence HSPs (e.g. parasites, predators or food availability) are modified in urban environments (Gil and Brumm, 2014). Besides potential differences existing between physiological variables and species (see above), these inconsistencies may be explained by the lack of replication (i.e. comparison of multiple cities). In fact, the majority of the studies assessing the effects of urbanization on vertebrate physiology are typically carried out in a single population (e.g. Chávez-Zichinelli et al., 2010; Fokidis et al., 2008; reviewed in Bonier, 2012), with very few including within-species comparisons or meta-replication at multiple urban and non-urban populations (Bonier et al., 2007b).

In this study, we measured multiple indicators of physiological stress at the endocrine, immune and cellular level (CORT$_f$, H/L ratio and HSP70, respectively) in five urban and five non-urban European blackbird (*Turdus merula*) populations. Birds are ideal for our approach because their physiology is relatively well studied and similar to that of other vertebrates (Hill et al., 2016). Furthermore, they have been widely studied within the urban ecology context (Gil and Brumm, 2014; Lepczyk and Warren, 2012; J. Marzluff et al., 2001; Murgui and Hedblom, 2017), and the blackbird has been proposed as an emerging model species for the study of urban-associated effects (Evans, 2010), thus offering key information to interpret physiological changes associated with urbanization. The specific research questions we wanted to test were: (1) whether urban blackbirds show higher levels of our target physiological traits (i.e. indicating poorer physiological condition) compared to conspecifics from natural habitats; (2) whether the effect of urbanization differs between physiological traits representing different levels of response (i.e. endocrine, immune or cellular); and (3) whether the patterns found are consistent among cities. We expect this study to shed new light on our understanding of the potential physiological impacts of urbanization on organisms.

2. Material and methods

2.1. Study design and sampling

We sampled adult blackbirds from ten different populations across Europe using a paired study design (Fig. 1). Each pair of locations (dyad) included an urban and a forest blackbird population separated on average (±s.e.) by 29.8 ± 3.8 km. This distance is an order of magnitude greater than the mean adult and natal dispersal distance of blackbirds (Paradis et al., 1998). We selected forest habitats as non-urban areas because forests are the ancestral habitat for this species (Luniak

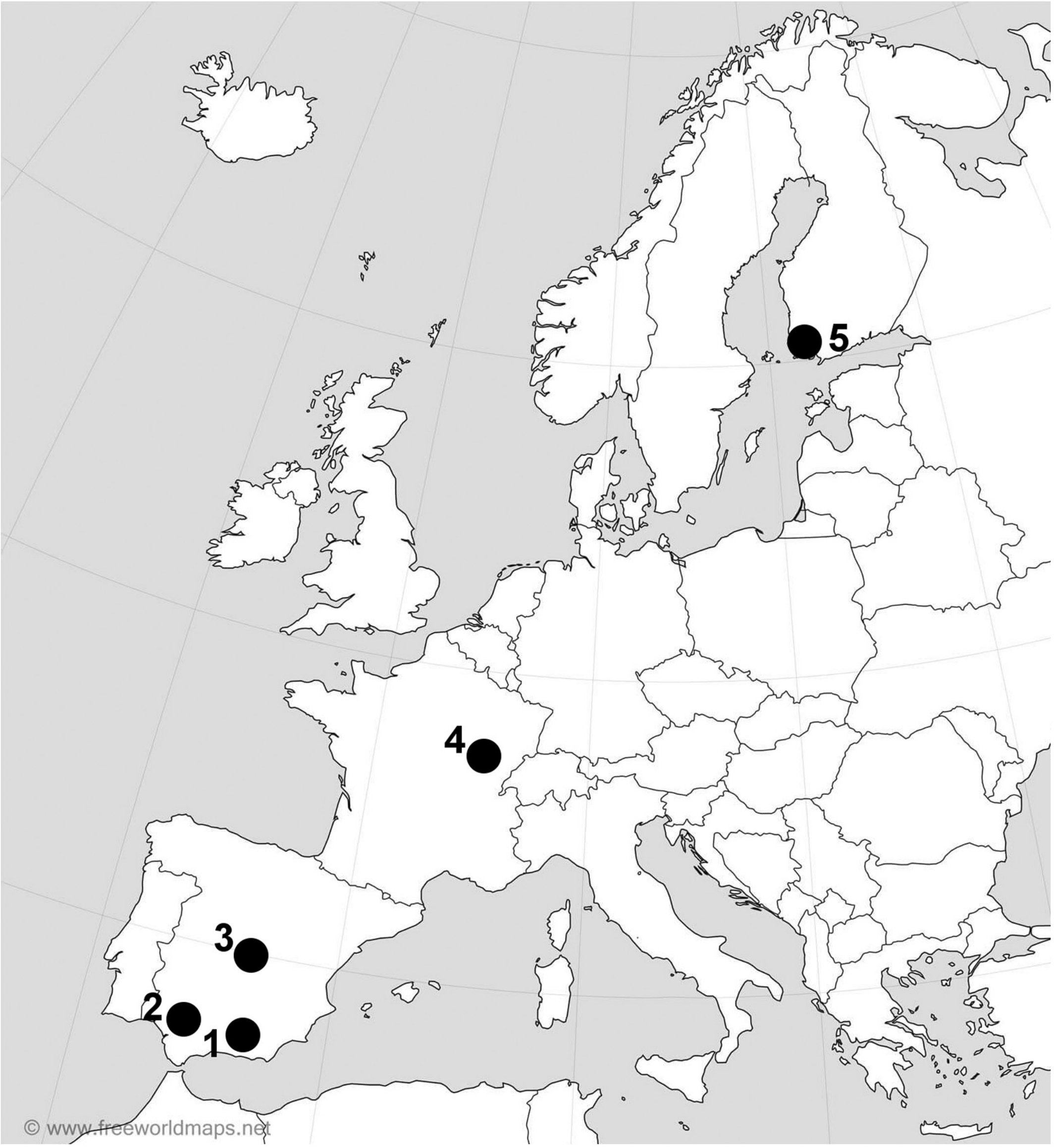

Fig. 1. Geographical distribution of the paired populations (dyads) included in the study: Granada (1), Seville (2), Madrid (3), Dijon (4), Turku (5). At each dyad we collected samples from an urban and a forest area separated by an average distance of 29.8 ± 3.8 km (mean ± s.e.).

et al., 1990). Urban capture sites were parks and gardens located in the core area of the cities, while forest sites were in forested protected areas with minimum human activity, in accordance with the definition of urban and wildland area proposed by Marzluff (2001). This habitat differentiation is also supported by a commonly used urbanization index (Liker et al., 2008) as shown in Ibáñez-Álamo et al. (2018).

Blackbirds, an urban adaptor species (Kark et al., 2007), were captured using mist nets in March–July 2015 (their main breeding season). Using plumage criteria (Jenni and Winkler, 1994), all captured birds were sexed and were classified as either yearlings (i.e. one year old individuals), or as ≥2 years old birds. Immediately after capture, blackbirds were bled from the brachial vein (350–450 μl). Blood samples were kept at 4 °C in a portable fridge for up to 5 h until centrifugation in the lab (5000 *g* for 15 min). We separated red blood cells from the plasma and froze them at −80 °C until analyses of heat-shock proteins were performed. We used a drop of blood to make blood smears that were air-dried in the field and later (within 5 h) fixed in methanol for 5 min. Just after blood extraction, we also collected the 6th secondary wing feather of the left wing of each captured blackbird and stored it in an individual envelop until lab analyses. Secondary feathers are molted in the breeding area during the post-breeding molt in blackbirds, and rarely during the post-juvenile molt (Jenni and Winkler, 1994). Thus, the CORT data obtained from these samples provided us with information on the period of feather growth during the previous year.

2.2. Lab analyses

2.2.1. Corticosterone

Hormone assays were carried out in the Spanish National Museum of Natural Sciences (Madrid, Spain). We quantified corticosterone levels in feathers ($CORT_f$ hereafter) following Bortolotti et al. (2008) with slight modifications. We removed the calamus from each feather and the rachis and feather vanes were minced into small pieces (<5 mm). We weighed each sample to the nearest 0.0001 g in a FX + 40 analytical balance (A&D Company Limited). After adding 6 ml of HPLC-grade methanol to the samples, we placed them into a sonicating water bath at room temperature for 30 min, followed by overnight incubation in a shaking water bath at 50 °C. We then used a nylon syringe filter (0.45 μ) to separate the methanol from the feather remains, which were washed twice with 2 ml of methanol to recover residual extracts. We placed the methanol solution into a water bath at 50 °C where the methanol was evaporated under a stream of nitrogen. Dry extracts were re-suspended in 150 μl of steroid-free serum (DRG, Germany) and vortexed for 10 min. $CORT_f$ levels were quantified with a commercial ELISA kit (DRG EIA-4164, Germany), following manufacturer instructions. We randomly include samples from multiple populations (mean = 3.5) in every plate to minimize the influence of the inter-assay variability in our findings. The inter-assay variability of our analyses was 12.76% while the intra-assay variability was 9.35%.

2.2.2. Heat-shock proteins

Among this group of proteins, we quantified levels of HSP70 because it is the most conserved protein in evolution and is very affected by multiple stressors (Daugaard et al., 2007; Kregel, 2002). Analyses of HSP70 were carried out at the Ecophysiology Laboratory at Estación Biológica de Doñana (Seville, Spain). HSP70 levels in red blood cells were quantified with a commercial ELISA kit (ADI-EKS-700B, ENZO Biochem Inc., Farmengdale, New York) following manufacturer instructions. Total proteins were measured using the Bradford method (Kruger, 1994) and HSP70 values were corrected according to total protein concentration in the samples.

2.2.3. Heterophils/lymphocytes ratio

Blood smears were stained using the Wright-Giemsa method (Brown, 1993) and sections with a monolayer of blood cells were scanned using a light microscope. The same observer (J.C.C.) randomly selected 100 leukocytes from each blood smear and classified each of them into heterophils, lymphocytes or others (e.g. monocytes, eosinophils or basophils). We calculated the H/L ratio dividing the number of heterophils by the number of lymphocytes.

2.3. Statistics

We tested the effect of urbanization on the three physiological variables analyzed: $CORT_f$, H/L ratio and HSP70, by fitting general linear mixed models with individual blackbirds as the sampling unit. None of the three response variables were significantly correlated with each other (all $r < 0.08$ and p-values >0.25) suggesting they reflect different characteristics of the individuals´ physiology. The full initial models included the following predictor variables: habitat (forest vs. urban), sex and age (yearlings vs. older birds, see methods). The two-way interactions including “habitat” (i.e. habitat x sex and habitat x age) were also included to test for a potential sex or age dependence of the effect of urbanization. Population nested within dyad was included as random factor, and for the $CORT_f$ model, we also included plate identity as random factor ($N = 9$). We used a backward selection process in which we sequentially removed the least significant terms until only significant terms ($P < 0.05$) remained in the model. After model selection, the Akaike information criterion (AICc; Burnham and Anderson, 2002) was also considered to confirm that the final models had the lowest AIC values. Logarithmic transformations (ln) were performed to normalize the three dependent variables, and residuals of the final models showed normal distributions.

One of the aims of this study was to investigate general effects of urbanization, and to what extent such effects were uniform across populations. Therefore, we further investigated the variability of our results across our 5 study dyads. We ran additional general linear mixed models in which dyad was included as fixed factor, and tested for the effect of its interaction with habitat as a predictor of $CORT_f$, H/L ratio and HSP70. These models included population as a random factor and, in the case of $CORT_f$, also plate ID, as well as the fixed factors of the final model for each response variable.

All statistical analyses were performed using R version 3.5.0 (R Core Team, 2018) and the R packages ‘lme4’ (Bates et al., 2014) and ‘lmerTest’ (Kuznetsova et al., 2016). We used the function r.squaredGLMM from the R package ‘MuMIn’ (Barton, 2018) to calculate the variance explained (R^2) by the random terms of our models. Tukey posthoc tests for pairwise comparisons were calculated with the R package ‘emmeans’ (Lenth et al., 2018).

3. Results

A total of 259 blackbirds were captured for this study with an average of 26.3 birds (SD = 6.4) per locality. The final sample size for each measurement differed slightly due to small sample volumes or logistical problems during the analyses in the laboratory: $CORT_f = 245$, $HSP70 = 249$, and H/L ratio = 235.

The backward and AICc selection procedures offered comparable results. The best model for $CORT_f$ using both methods included Habitat, Sex and the interaction between these two terms (Tables 1 and S3). More specifically, $CORT_f$ levels were significantly lower in urban blackbirds compared to non-urban conspecifics (Table 1). However, this effect of urban habitat on $CORT_f$ was marginally affected by sex (Habitat x Sex: $F_{1,233.0} = 3.82$; $p = 0.052$), with only male blackbirds showing differences between habitats (Fig. 2). In contrast, we did not find a significant effect of habitat on HSP70 levels or H/L ratio nor of any of the other terms included in the models (all p-values >0.18; Tables S1, S2), which was confirmed by the AICc results as the null model was also the best model (Tables S4 and S5).

When testing for the geographic variation in the effect of urbanization (i.e. interaction between habitat and dyad), we found a significant

Table 1
General Linear Mixed Model exploring habitat differences (urban vs forest) in blackbird feather corticosterone (pg/mg; ln transformed). *P*-values lower than 0.05 are shown in italics. Sample size: 245 individuals. Final model $R^2 = 0.59$.

	Estimate	SE	Df	F	P
Intercept	2.24	0.18			
Habitat (Urban)	*−0.46*	*0.12*	*1, 5.4*	*10.53*	*0.021*
Sex (Female)	−0.02	0.08	1, 234.0	2.50	0.116
Sex (Female) × Habitat (Urban)	0.22	0.11	1, 233.0	3.82	0.052
Rejected terms					
Age (Old)	−0.07	0.06	1, 232.4	1.31	0.254
Age (Old) x Habitat (Urban)	−0.10	0.12	1, 233.8	0.69	0.407
Random factors			**Variance**		
Plate ID			0.214		
Dyad			0.009		
Population:Dyad			0.015		
Residual			0.189		

effect for CORT$_f$ ($F_{4,222.9} = 2.86$, $p = 0.02$; Fig. 3A) and HSP70 ($F_{4,259} = 4.78$, $p < 0.0001$; Fig. 3B) but not for H/L ratio ($F_{4,235} = 1.41$, $p = 0.23$; Fig. 3C). Thus, the effect of urbanization significantly differed between paired populations for two of our three physiological indicators. These differences between dyads, however, were not consistent among the three variables, which overall showed dissimilar patterns (Fig. 3). Furthermore, in the models including population nested within dyad as random factor, the proportion of explained variance by this random factor differed greatly for each physiological indicator (CORT$_f$ = 38%; HSP70 = 13%; H/L ratio = 9%).

4. Discussion

Our results suggest that living in urban environments has varying effects on the stress physiology of an urban-adaptor species, the European blackbird. We found consistent differences between urban and non-urban habitats only in CORT$_f$, and this effect was only observable in males, with urban males showing lower CORT$_f$ values. The disparity of patterns among variables indicates that there are a variety of mechanisms of physiological response to potential stressors that do not show correlated responses (Romero and Wingfield, 2016). Thus simplistic approaches may fail to hit the correct parameter and will require looking at a variety of physiological variables to understand how organisms respond to urbanization pressures. Including multiple physiological variables is important to form an overall picture of the effect of different environmental factors on the physiological state of animals (Matson et al., 2006; Müller et al., 2011). Furthermore, for two of our physiological stress markers (CORT$_f$ and HSP70), the strength and even the direction of the effect of urbanization significantly differed among paired populations, indicating that local effects mediate urban-induced physiological changes. This complex physiological response to urbanization found in several populations of the same species could also partly explain the contrasting results of previous studies investigating physiological stress in relation to urbanization in birds (Bonier, 2012).

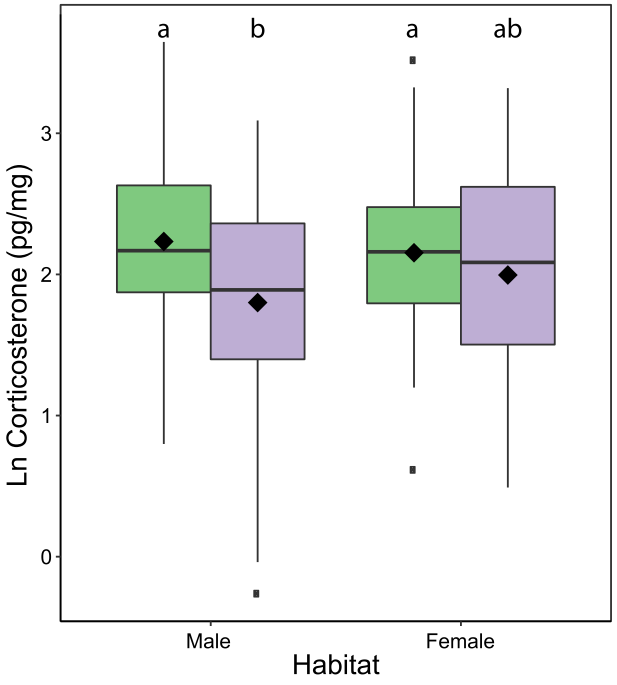

Fig. 2. Blackbird feather corticosterone levels in relation to sex for urban (purple) and forest (green) habitats. The boxplots show the mean (rhombus), median (bar inside the rectangles), upper and lower quartiles and extreme values. Different letters above boxplots indicate significant differences according to Tukey posthoc tests.

Overall, our results suggest that urban habitats are not detrimental for this bird species in terms of physiological stress. This finding might seem surprising given that urban adult blackbirds have shorter telomeres than forest ones (Ibáñez-Álamo et al., 2018), and telomeres are considered as biomarkers of cumulative physiological damage in many organisms, including the blackbird (Hau et al., 2015). However, it is possible that these differences in telomeres arise before adulthood (see Ibáñez-Álamo et al., 2018), which would be supported by data showing that nestling blackbirds raised in the city suffer a higher starvation risk than in the forest (Ibáñez-Álamo and Soler, 2010). This nutritional challenge during development has also been suggested as a crucial factor underlying the reduced stress responses of urban-born blackbirds (Partecke et al., 2006). Taken together, these results suggest that the urban-induced effects on blackbird physiology depend on the life history stage, which may explain the apparent inconsistency between the results on telomeres and those on physiological stress. Alternatively, it is also possible that telomere shortening is due to stressors not reflected by the three physiological parameters studied.

We found significant differences in CORT$_f$ between urban and non-urban blackbirds, as stated above. These results showed lower CORT$_f$ values in urban individuals compared with forest dwellers. To our knowledge, there are only two previous studies testing for differences in CORT$_f$ between urban and non-urban habitats (Beaugeard et al., 2019; Meillère et al., 2016). Both studies found higher CORT$_f$ in urban birds, but the only one including multiple geographical locations (Beaugeard et al., 2019) was done on house sparrows (*Passer domesticus*) and included juveniles only. Several local studies on plasma CORT support our finding, showing lower baseline (Wright and Fokidis, 2016) and stress-induced (Partecke et al., 2006; Wright and Fokidis, 2016) plasma CORT concentrations in urban birds. In contrast, other studies reported no overall differences in baseline plasma concentrations between urban and non-urban birds (H. Bobby Fokidis et al., 2009; Foltz et al., 2015; Grunst et al., 2014; Hudin et al., 2018; Injaian et al., 2020; Meillère et al., 2015), including captive blackbirds (Partecke et al., 2006). There are several potential explanations for these differences between our findings and those of previous studies. On the one hand, several studies concluded that the relationship between urbanization and CORT varies across species and contexts (i.e. cities) (Bonier, 2012; H. Bobby Fokidis et al., 2009; Injaian et al., 2020; this study). On the other hand, CORT$_f$ seems to better reflect variation in the acute stress response rather than variation in baseline CORT (Beaugeard et al., 2019), which would explain why our results generally match better those on stress-induced levels (Partecke et al., 2006). In addition to this variation in response to urban environments, CORT is known to mobilize body reserves (i.e. glucose, fatty acids and proteins; Jimeno et al., 2018a, 2018b; Remage-Healey and Romero, 2001; Sapolsky et al., 2000) to provide the resources needed to cope with current or anticipated energetic needs (Jimeno et al., 2018a; McEwen and Wingfield, 2003). Therefore, CORT concentrations are expected to

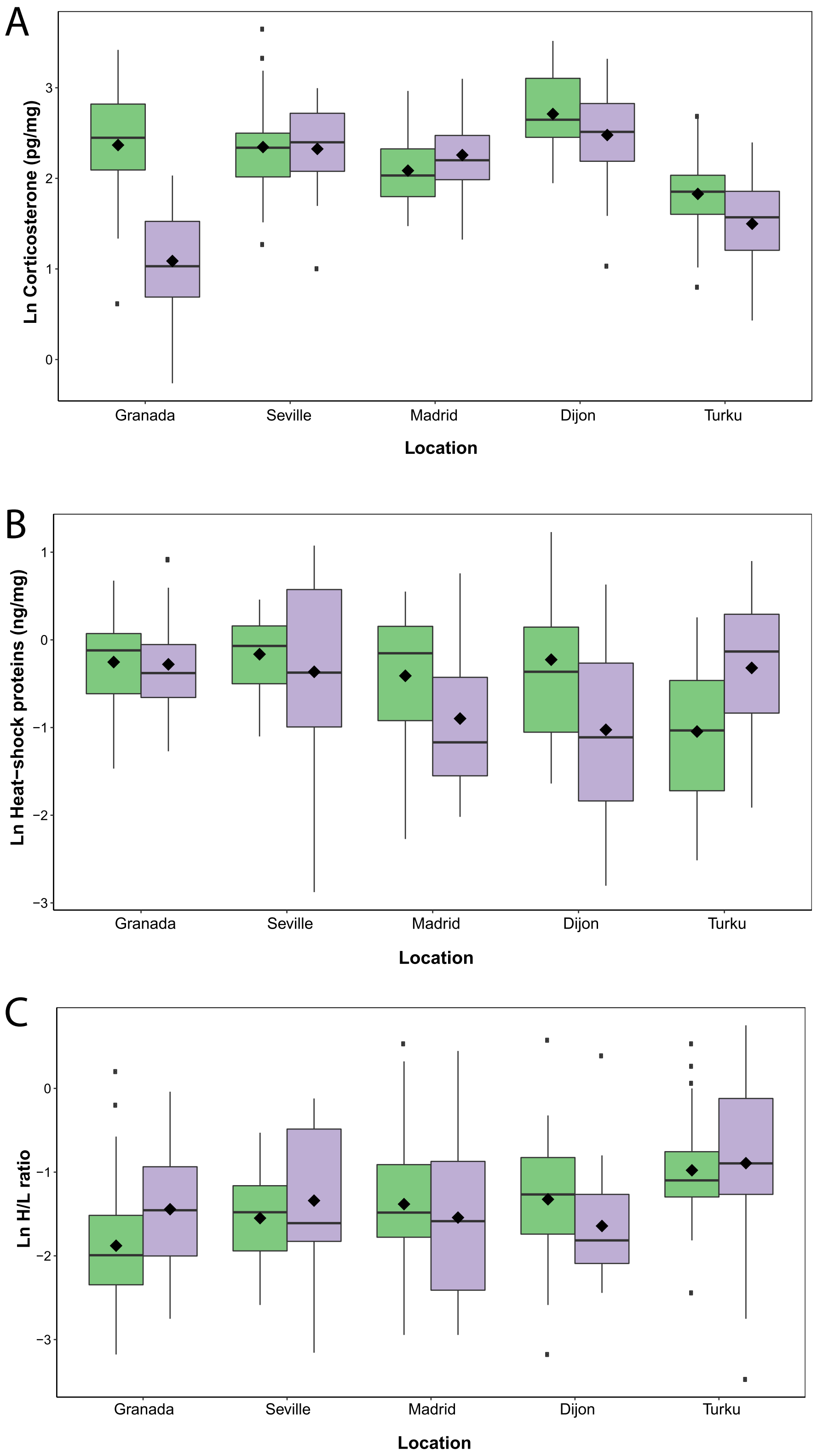

Fig. 3. Blackbird feather corticosterone (A), heat-shock protein 70 levels (B) and heterophils to lymphocytes (H/L) ratio (C) by paired population (dyad) for urban (purple) and forest (green) habitats. The boxplots show the mean (rhombus), median (bar inside the rectangles), upper and lower quartiles and extreme values.

increase in energetically demanding circumstances, such as high foraging costs (Jimeno et al., 2018b). Urban environments may be an easy foraging environment for adult blackbirds, with lower energy expenditure needed to access resources compared to non-urban areas. In addition, diet composition can also play a role in the concentrations of circulating CORT (Cano et al., 2008). Urban and non-urban bird populations differ in their diet composition (e.g. fatty acids; Isaksson et al., 2017; Meillère et al., 2015), and these differences may drive changes in the composition and availability of energy reserves, and eventually in circulating CORT levels. Alternatively, urban blackbirds may show lower CORT values as a consequence of chronic stress, as unlike with acute stressors (where CORT levels are assumed to increase), chronic stress can lead to both hypo- or hyper-regulation of CORT (reviewed in Dickens and Romero, 2013). However, we consider this latter hypothesis unlikely because we did not find overall differences between urban and forest individuals in any of the other variables that we measured.

Interestingly, the effect of urbanization on $CORT_f$ seems to be marginally dependent on sex, being only significant in males. The majority of studies on the topic have not identified sex differences in CORT levels associated to urbanization (Injaian et al., 2020). Only two studies have found sex-specific changes in (baseline) CORT between urban and non-urban individuals, but offering contrasting results. One of them, on white-crowned sparrows (*Zonotrichia leucophrys*), found higher levels of baseline CORT in urban compared with non-urban males, while no differences were observed in females (Bonier et al., 2007a). In contrast, American kestrels (*Falco sparverius*) females, but not males, showed higher baseline CORT levels while breeding in urban habitats compared with those reproducing in less disturbed areas (Strasser and Heath, 2013). This suggests that there could be species-specific traits behind these sex-specific relationships between urbanization and CORT. These studies (including ours) were carried out during the breeding season, and therefore it is likely that these sex differences arise because of habitat-associated differences in female vs male physiology during reproduction (e.g. related to activities like incubation, chick feeding or territory defence).In the specific case of blackbirds, it seems that urban and non-urban males differ in the timing of gonadal growth and migratory disposition, while females do not show such pattern (Partecke and Gwinner, 2007; Partecke et al., 2004). This would be consistent with our findings, indicating that male and female blackbirds regulate part of their physiology differently in urban and non-urban environments.

In contrast to $CORT_f$, we did not find evidence of an overall effect of urbanization on blackbird H/L ratios or HSP70 plasma levels. This finding is consistent with other urban exploiter and adaptor bird species also showing no significant variation between urban and non-urban areas in H/L ratio (Bókony et al., 2012; Carbó-Ramírez and Zuria, 2017; H. B. Fokidis et al., 2008). Taken together, our findings suggest a general trend among those species benefiting from these human-induced habitats. In contrast, urban-avoider or less-urban adaptable species show increased H/L ratios in urban habitats (H. B. Fokidis et al., 2008), although additional studies using different species and sites will be needed to confirm this pattern. H/L ratio has been interpreted as an indicator of immune challenge (Cirule et al., 2012; Davis et al., 2004; Krams et al., 2012), and therefore our results suggest that urban blackbirds are not more immunologically challenged than forest blackbirds. However, two recent studies using transcriptome analysis found an elevated expression of some immune genes in urban populations of two tit (*Paridae*) species (Capilla-Lasheras et al., 2017; Watson et al., 2017) which points at more complex urban-induced immune effects, the need to obtain a broader view of the immune system using additional variables, and to consider species ranging from adaptors to avoiders. Our results for HSP70 would also be in line with those of $CORT_f$ and H/L ratio, also indicating that urban habitats are not physiologically challenging environments for this species. Given the negative association between HSP70 levels and food availability (Herring et al., 2011), the lack of differences in urban and non-urban European blackbirds could suggest that cities do not represent habitats with limited food resources for adult blackbirds. This would also match with the previous interpretation on $CORT_f$ levels and its association with foraging costs (see above).

The absolute values of $CORT_f$, HSP70 and H/L ratio, as well as the strength and direction of urbanization effects on these variables showed wide variation among European cities. This broad geographical variation could partly explain contradicting results reported by previous studies investigating the effects of urban vs. non-urban habitat on avian physiology (Bonier, 2012), as most of them were focused in one single city (e.g. Chávez-Zichinelli et al., 2010; Partecke et al., 2006; Ruiz et al., 2002). We do not know the causes of such variation but it is possible that differences among cities in a variety of environmental (e.g. number or type of green areas, proximity to the sea, humidity or precipitation) and human factors (e.g. population density, pollution) may explain these differences (e.g. Bauerová et al., 2017; Treen et al., 2015). Despite the origin of such geographical variation, our results stress the importance of including spatial replicates in multiple cities to infer widespread patterns when studying the ecological consequences of urbanization (Ibáñez-Álamo et al., 2018).

To our knowledge, this is the first study investigating multiple indicators of physiological stress (hormonal, immunological and cellular) among multiple locations in an urbanization context. Our findings suggest that living in cities is not physiologically detrimental for European blackbirds, but species differing in ecology and life history strategies (i.e. urban avoiders) may likely differ in their physiological capacity to cope with urban-associated changes (see Bonier, 2012; Fokidis et al., 2008). Given the variation in results offered by the different physiological variables and different geographical locations, our work also highlights the importance of relying on multiple physiological variables and spatial replicates to assess the impact of urbanization on animal physiology. Large scale and multilevel approaches will also be crucial to infer general patters in the study of urbanization consequences, providing key information to implement effective management and city- or species-specific conservation plans.

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CRediT authorship contribution statement

Juan Diego Ibáñez-Álamo: Conceptualization, Methodology, Validation, Formal analysis, Investigation, Resources, Writing - original draft, Visualization, Supervision, Project administration, Funding acquisition. **Blanca Jimeno:** Validation, Formal analysis, Writing - original draft. **Diego Gil:** Formal analysis, Resources, Writing - review & editing. **Robert L. Thomson:** Investigation, Resources, Writing - review & editing. **José I. Aguirre:** Investigation, Writing - review & editing. **Alazne Díez-Fernández:** Investigation, Writing - review & editing. **Bruno Faivre:** Investigation, Writing - review & editing. **B. Irene Tieleman:** Resources, Supervision, Funding acquisition. **Jordi Figuerola:** Investigation, Resources, Writing - review & editing, Supervision, Funding acquisition.

Declaration of competing interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scitotenv.2020.137332.

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