

# 1 **Testing for parallel genomic and epigenomic footprints of** 2 **adaptation to urban life in a passerine bird**

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24

25 **Abstract (250 words):**

26 Identifying the molecular mechanisms involved in rapid adaptation to novel environments and  
27 determining their predictability are central questions in Evolutionary Biology and pressing issues due to  
28 rapid global changes. Complementary to genetic responses to selection, faster epigenetic variations such  
29 as modifications of DNA methylation may play a substantial role in rapid adaptation. In the context of  
30 rampant urbanization, joint examinations of genomic and epigenomic mechanisms are still lacking.  
31 Here, we investigated genomic (SNP) and epigenomic (CpG methylation) responses to urban life in a  
32 passerine bird, the Great tit (*Parus major*). To test whether urban evolution is predictable (*ie* parallel)  
33 or involves mostly non-parallel molecular processes among cities, we analysed three distinct pairs of  
34 city and forest Great tit populations across Europe. Results reveal a polygenic response to urban life,  
35 with both many genes putatively under weak divergent selection and multiple differentially methylated  
36 regions (DMRs) between forest and city great tits. DMRs mainly overlapped transcription start sites and  
37 promotor regions, suggesting their importance in the modulation gene expression. Both genomic and  
38 epigenomic outliers were found in genomic regions enriched for genes with biological functions related  
39 to *nervous system, immunity, behaviour, hormonal and stress responses*. Interestingly, comparisons  
40 across the three pairs of city-forest populations suggested little parallelism in both genetic and epigenetic  
41 responses. Our results confirm, at both the genetic and epigenetic levels, hypotheses of polygenic and  
42 largely non-parallel mechanisms of rapid adaptation in new environments such as urbanized areas.

43 **Significant statement (120 words):**

44 Urbanization drives, all around the globe, tremendous changes in the ecology of species and in  
45 individual phenotypes. Molecular bases of phenotypic shifts and of adaptation to urban environments  
46 remain under-explored. In particular, the roles of genetic and epigenetic mechanisms, and their  
47 parallelism across cities, are still unknown. We searched for genomic (SNP markers) and epigenomic  
48 (CpG methylation) differences between urban and forest populations of great tits in three European  
49 locations. We identified several and mostly non-parallel molecular marks associated with urbanization.  
50 These marks were however associated to similar biological functions related to the nervous system,  
51 behaviour and stress response. This study suggests important roles of *de novo* genetic and epigenetic  
52 variation during adaptation to life in the city.

## 53 INTRODUCTION

54 Identifying mechanisms involved in rapid adaptation to new environmental conditions is a  
55 central question in evolutionary biology and a is pressing task in the context of global changes of the  
56 Anthropocene (1). The vast majority of studies investigating mechanisms involved in rapid adaptation  
57 to new environments have focused on phenotypic plasticity on the one hand and on genetic response to  
58 selection on the other hand. At their crossroad, recent work underlines the potential role of epigenetics  
59 in rapid adaptation to new environments (2). In particular, environmental variations can induce  
60 differences in DNA methylation patterns and hence modulate genes' expression and upper-level  
61 phenotypes (3, 4). Such methylation-linked phenotypic variations can occur during an individual's  
62 lifetime, especially early on during the organism's development (5, 6). Although methylation changes  
63 acquired across an individual's lifetime may often be non-heritable (7 ; but see 8, 9), epigenetically  
64 induced phenotypic shifts may nevertheless enhance individuals fitness in new environments. Moreover,  
65 during the course of evolution, divergent genetic variants regulating epigenetic modifications may also  
66 be selected for, hence promoting the evolution of divergent epigenotypes and epigenetically-linked  
67 phenotypic variation (10). While epigenetic studies focused on human diseases and medical topics are  
68 now abundant, studies in an ecological context are still rare (11). Nevertheless, a few epigenetic studies  
69 in natural populations revealed that DNA methylation shifts might play a determinant role in local  
70 adaptation to environmental variation (12). There is hence an urgent need for further empirical  
71 investigations of simultaneously rapid genetic and epigenetic evolution in response to environmental  
72 change (13).

73 Urbanization rapidly and irreversibly changes natural habitats into human-made environments  
74 and is considered as a major threat to biodiversity (14). For species who appear to cope with  
75 urbanisation, urban habitats present a myriad of novel environmental conditions compared to the habitat  
76 where they evolved, including high levels of chemical, light and sound pollution, high proportion of  
77 impervious surfaces, high habitat fragmentation, low vegetation cover and high human densities (15,  
78 16). Such extreme environmental changes compared to natural areas are expected to result in numerous  
79 new selection pressures on city-dwelling species (17). Accordingly, rates of recent phenotypic change,  
80 concerning multiple types of traits related to behaviour, morphology, phenology and physiology, seem

81 to be greater in urban areas than in any other habitat types, including non-urban anthropogenic contexts  
82 (18, 19). The exploration of the molecular mechanisms implicated in urban-driven phenotypic changes  
83 has only begun, with both genetic (20–22), and epigenetic investigations (23–25). For instance, DNA  
84 methylation variations have been associated in vertebrates with high levels of traffic-related air pollution  
85 (e.g. 26). Yet, epigenetic studies have been performed at relatively small genomic resolution. In addition,  
86 very little is known about the level of parallelism and hence of the predictability of genetic and  
87 epigenetic evolution in response to urbanisation in distinct cities (27, 28). So far, there are situations  
88 ranging from local adaptation despite strong gene flow (e.g. in the red-tailed bumblebee *Bombus*  
89 *lapidaries*, 29) to restricted gene flow and independent colonization in different cities by a few founders,  
90 followed by adaptation (e.g. in the burrowing owl *Athene cunicularia*, 30). Providentially, recent  
91 genomic tools and virtually limitless amount of cities offer unique opportunities for comparing at high  
92 genomic resolution simultaneously individuals' genomic and epigenomic responses in several cities to  
93 study the parallelism and predictability in molecular mechanisms implicated in rapid adaptation to  
94 urbanization (31, 32).

95 In this study, we used genome-wide and epigenome-wide sequencing approaches to compare  
96 genetic and epigenetic responses among three pairs of great tit *Parus major* populations in urban and  
97 forest habitats. At the European level, population monitoring of Great tits revealed parallel phenotypic  
98 shifts in city birds compared to their forest conspecifics, with in particular smaller and lighter urban  
99 birds laying earlier and smaller clutches (33–36). We investigated both average genomic (SNPs) and  
100 epigenomic (CpG methylation) differentiation and we searched for particular genomic footprints of  
101 divergent selection as well as differentially methylated regions, between forest and urban populations.  
102 Our results show that despite limited genetic differentiation and few genomic footprints of divergent  
103 selection between forest and urban populations, urban life was associated with numerous differentially  
104 methylated regions notably associated with neural development, behaviour and immunity. Hence, this  
105 study shows the potential role of epigenetic response in rapid adaptation to changing environments in  
106 urban areas. Importantly, we found little parallelism between cities in both genomic and epigenomic  
107 responses to urbanization, possibly confirming the hypothesis that multiple evolutionary ways exist to  
108 independently cope with similar novel environmental conditions.

109

## 110 RESULTS

### 111 Little genetic and epigenetic average differentiation between urban and rural populations

112 **Genetic.** We used a redundancy analysis (RDA) on 74,137 SNPs obtained by RAD-sequencing to  
113 document genetic variation among the studied great tits populations, with location (Barcelona,  
114 Montpellier or Warsaw), habitat (urban vs. forest), and sex as explanatory variables. The model was  
115 highly significant ( $P < 0.001$ ) but explained only a small fraction (*i.e.* less than 2%) of the total variance:  
116  $R^2 = 0.018$  (Figure 1C, SI Table S1). All three variables were significant (location:  $P = 0.001$  ; habitat:  
117  $P = 0.004$ ; sex:  $P = 0.001$ ). Partial RDA revealed that the net variation explained by the habitat  
118 ( $R^2 = 0.004$ ,  $P = 0.001$ ) was inferior to the net variation explained by location ( $R^2 = 0.012$ ,  
119  $P = 0.001$ ) but higher than sex ( $R^2 = 0.002$ ,  $P = 0.004$ , Table S1). As expected, when removing the  
120 Z chromosome from the data, sex became non-significant ( $P = 0.260$ ), whereas the effects of other  
121 variables remained significant and of similar magnitude (Table S2).

122 Genome-wide differentiation between populations, measured with Weir and Cockerham's  $F_{ST}$   
123 (37) was relatively low on average (mean  $F_{ST} = 0.019$ ), in the order of 1 to 2% between habitats for  
124 each location ( $F_{ST}^{Barcelona(rur-urb)} = 0.018 \pm 0.001$  ;  $F_{ST}^{Montpellier(rur-urb)} = 0.012 \pm 0.001$  ;  $F_{ST}^{Warsaw(rur-urb)}$   
125  $= 0.018 \pm 0.001$ ) (see SI Table S3), suggesting relatively high gene flow and limited genetic drift among  
126 populations. Mean  $F_{ST}$  on autosomes was lower (mean  $F_{ST} = 0.014$ , Table S4) than on the Z chromosome  
127 (mean  $F_{ST} = 0.022$ , Table S5).

128 **Methylation.** Similarly to genetic data, we performed an RDA on methylation level of 157741 CpG  
129 sites to describe epigenetic variation among individuals in relation to location, habitat and sex. The  
130 model was significant but explained less than 1% of the total variance ( $R^2 = 0.007$ ,  $P = 0.001$ ). All  
131 variables contributed significantly (location :  $P = 0.001$ , habitat:  $P = 0.03$ , sex:  $P = 0.001$ , Table S6).  
132 Partial RDA revealed that location and sex explained a similar proportion of the total variance which  
133 was higher than habitat (location:  $R^2 = 0.003$ ,  $P = 0.001$ ; sex:  $R^2 = 0.003$ ,  $P = 0.001$ ; habitat:  $R^2 = 0.001$ ,  
134  $P = 0.3$ ). When removing the Z chromosome from analyses, results remained similar (Table S7),

135 showing that difference in methylation was not entirely driven by potential diverging patterns associated  
136 with sexual chromosomes.

137 We then investigated more finely whether individual methylation on CpG cytosines varied  
138 across location, habitat (urban vs forest), sex, and location×habitat interaction, using an ANOVA, run  
139 on autosomes and Z chromosome separately (Fig 1B, Table S8). For autosomes, we detected a  
140 significant effect of location ( $F = 3.319$ ,  $P = 0.044$ ), with Montpellier individuals showing lower  
141 methylation levels than Warsaw ones (Fig 1B, Tukey test:  $P = 0.04$ ) and no other difference between  
142 pairs of cities. Also, no significant effect of sex ( $P = 0.263$ ) or habitat ( $P = 0.478$ ) was found, suggesting  
143 that urbanization did not have an important overall effect on global methylation levels. For the Z  
144 chromosome, we found a strong difference between sexes, with homogametic males showing 2.98%  
145 more methylated Z than heterogametic females (Fig 1B;  $P = 1.45 \times 10^{-15}$ ), while no significant difference  
146 between location ( $P = 0.577$ ) or habitat ( $P = 0.915$ ) was found.

147

#### 148 **Non-parallel yet strong genomic footprints of divergent selection between urban and forest** 149 **populations and evidence for polygenic adaptation**

150 We used two methods to investigate outlier SNPs potentially under divergent selection between  
151 forest and urban populations: an  $F_{ST}$ -outlier based method (v2.1, (38), default parameters), and a  
152 multivariate method (using an RDA, (39)) aiming respectively at identifying strong outliers indicating  
153 footprints of differentiation for each population pair and weaker outliers across the several population  
154 pairs studied at once. First, Bayescan identified 15 outliers for Barcelona, 11 for Montpellier and 10 for  
155 Warsaw, overall distributed on 15 chromosomes and associated to 13 genes in 5kb upstream or  
156 downstream regions (Figure 2)( $q\text{-value} < 0.1$ , see Figure S1). None of these outliers was shared between  
157 the three population pairs, revealing no convergence between cities. Second, using the multivariate  
158 approach based on a RDA (following the procedure for constrained RDA described earlier), we  
159 investigated the habitat effect and extracted a list of 1163 loci with outlier loading score following  
160 Forester *et al.* (39) method (Figure 2, see Figure S2 for threshold), suggesting a higher number of loci  
161 undergoing weaker and polygenic selection. These 1163 loci were associated with 561 genes.

162 To investigate the potentially statistically enriched gene ontologies (GO) for the set of genes  
163 associated to RDA outliers, we used topGO R package (40) with GO referenced for the chicken *Gallus*  
164 *gallus* (ggallus\_gene\_ensembl). GO analyses revealed the existence of overrepresented ontologies ( $P <$   
165  $0.05$  and at least 3 genes per GO). Among the most promising GO terms we found functions related to  
166 the nervous system (GO:0048846, axon extension involved in axon guidance; GO:0035418 protein  
167 localization to synapse; GO:0021987 cerebral cortex development; GO:0007274, neuromuscular  
168 synaptic transmission), the blood system (GO:0045777 positive regulation of blood pressure;  
169 GO:0042311, vasodilatation), hormonal response (GO:0071277, cellular response to estrogen stimuli)  
170 and stress (GO:0033555, multicellular response to stress), revealing potential important functions  
171 involved in adaptation to urban habitats. Detailed GO results are presented in SI Table S9 and Figure  
172 S3.

173

#### 174 **Evidence for differentially methylated regions in urban environments**

175 We identified a total of 224 distinct DMRs between urban and forest great tits: 80 for Barcelona,  
176 68 for Montpellier and 93 for Warsaw. Only 14 DMRs (6.25%) were found repeatedly in at least two  
177 comparisons, and only 3 were common to the three cities. 7 (of these 14 parallel DMRs were in the same  
178 direction of methylation in urban compared to forest areas (Figure 3, Figure S4 A). Barcelona urban  
179 birds presented significantly more hypomethylated than hypermethylated DMRs ( $\chi^2 = 11.25$ ,  $P < 0.001$ ),  
180 but no difference was found for Montpellier ( $\chi^2 = 0.941$ ,  $P = 0.332$ ) nor for Warsaw ( $\chi^2 = 0.011$ ,  $P =$   
181  $0.917$ ). DMRs were distributed across all the 32 chromosomes as well as on 37 unplaced scaffolds. 203  
182 of the 224 different DMRs (91%) overlapped genes or 5kb flanking regions, among which 52% were  
183 directly located in gene bodies, promoter or TSS sequences (35.3% in gene bodies and 47.3% in  
184 promoters/TSS) suggesting their putative functional role in gene expression and regulation and/or  
185 splicing events.

186 Following the procedure previously described, GO analyses on the pooled genes list revealed  
187 an overrepresentation of modules associated to the nervous system (GO:2000300, regulation of synaptic  
188 vesicle exocytosis; GO:0050804 modulation of synaptic transmission), immunity (GO:005728, negative  
189 regulation of inflammatory response; GO:0050852, T cell receptor signaling pathway), metabolic

190 activity (GO:006816 calcium ion transport; GO:0055072, iron ion homeostasis, GO:0043087, regulation  
191 of GTPase activity), behaviour (GO:0007626, locomotory behavior) and endocrine processes  
192 (GO:0044060: regulation of endocrine process). All enriched GO are presented in SI Figure S5 and  
193 Table S10.

194 We also searched for DMRs between sexes, following the same procedure. We identified 206  
195 DMRs associated with sex, of which 58 for Barcelona, 81 for Montpellier and 99 for Warsaw. On a total  
196 of 206 DMRs, 181 (57.3%) were on genes or in a 5kb upstream/downstream region around genes. GO  
197 analyses revealed enrichment of genes involved in development, growth and morphogenesis, among  
198 others (see detailed enriched GO Table S10 & Figure S5).

199 Almost twice more sex DMRs were shared between locations (11,7%) than between habitats  
200 (6.25%, see Figure S4 A & B ; z-test:  $\chi^2$ -squared = 3.885, P = 0.049). When taking into account the  
201 direction of methylation difference, 7 sex DMRs were shared between at least two cities (9.7%) which  
202 was three times more than for habitat DMRs (3.1%; z-test: X-squared = 7.904, P = 0.005).

203

## 204 **DISCUSSION**

205 The urban sprawl is a worldwide phenomenon deeply affecting the environment and thus  
206 requiring fast adaptive responses in city dwellers. While a large body of literature already describes a  
207 myriad of phenotypic shifts in urban populations of numerous species (35, 41, 42), the molecular bases  
208 of these shifts and their evolutionary implications remain yet to be documented and understood. This  
209 study uses for the first time genomic and epigenomic analyses to decipher the potential molecular bases  
210 implicated in phenotypic shifts and adaptation in several urban populations of a passerine bird, the Great  
211 tit. Note that this species shows largely parallel phenotypic shifts across its range in terms of morphology  
212 and life history (Thompson *et al.*, *sub*). Genomic analyses revealed weak yet significant average  
213 differentiation between urban and forest populations, suggesting ongoing gene flow and limited drift in  
214 urban populations. These analyses also identified a limited number of loci putatively under strong  
215 selection, non-repeated between pairs, and numerous loci supposedly under weaker selection,  
216 compatible with a polygenic model of evolution. From the epigenomic side, we found weak average



217 differentiation of the methylome between urban and forest birds, suggesting an absence of genome-wide  
218 epigenetic deregulations, which is notably in line with an absence of strong genetic differentiation. In  
219 turn, we identified several strongly differentially methylated regions between urban and forest birds,  
220 mostly non-repeated between pairs and hence potentially implicated with local evolution of urban  
221 populations. Genes associated with either genomic footprints of divergent selection or differentially  
222 methylated regions had relatively similar functions, related to the nervous system, metabolism,  
223 immunity and behaviour, that have been repeatedly convicted in other studies (e.g. 40). Hence, by  
224 identifying non-repeated genetic and epi-genetic responses among replicated forest-urban population  
225 pairs, our findings support the hypothesis of mostly non-parallel rapid *de novo* adaptation to similar  
226 environments via both genetic and epigenetic mechanisms. Our results are in line with accumulating  
227 evidence that polygenic adaptation and epigenetic reprogramming may be involved in quick phenotypic  
228 shifts in response to rapidly emerging constraints such as urbanization.

229

230 Overall genetic differentiation between populations was relatively low ( $F_{ST}$  ranging from 0.9%  
231 to 3.4%), although higher than what has been found at a much larger scale across the species distribution  
232 (e.g. around 1% of  $F_{ST}$  between UK and Spanish or French and Spanish populations, 41). Low but  
233 significant differentiation levels are in line with previously documented divergences between city and  
234 forest great tit populations (20, 45), which altogether suggests important gene flow, large effective  
235 population sizes and limited genetic drift at multiple spatial scales (46). This overall genetic context is  
236 particularly suitable to search for genomic footprints of divergent selection between urban and forest  
237 populations, which would easily be identifiable above the neutral level of genetic differentiation.

238 We found a limited number of strong footprints of divergent selection, which is in line with  
239 previous results of Perrier et al (2018) in Montpellier, and of Salmon et al (2020) across nine European  
240 cities. Similarly to low levels of parallelism in allele frequency changes between cities observed by  
241 previous studies (22, 47), and despite similarities in phenotypic shifts in the European cities (e.g. 35),  
242 none of these outliers were shared between cities. This result suggests limited parallel evolution,  
243 supporting a scenario of independent *de novo* evolution between cities and/or different selection  
244 pressures between cities. Indeed, there may be multiple evolutionary solutions to the same

245 environmental challenges (48) and multiple traits are linked to the same functional outcome (49).  
246 Besides, the identification of numerous outliers by the multivariate framework applied at the scale of all  
247 six sampling sites supports a model of polygenic urban adaptation implicating multiple genes, biological  
248 pathways and phenotypic traits (50). Polygenic adaptation may be expected in urban habitats since the  
249 multiple new environmental conditions in cities most probably result in many novel selective pressures  
250 acting on a multitude of functional traits (51), and because many of these traits may be quantitative,  
251 and genetically correlated (52). Further polygenic analyses on more samples and more markers (i.e.  
252 whole genome data) are however required to increase our capacity to estimate more precisely the  
253 potential effect of each genetic variant implicated in the adaptation to life in the city (53, 54).

254         Several genomic footprints of divergent selection between urban and forest environments were  
255 in, or in the vicinity of, genes that have already been described as playing a role in neuronal development,  
256 behaviour or cognitive abilities. In particular, the NR4A2 gene plays an important role in recognition of  
257 novel objects and memory in mice (55). Reaction to novel objects and novel food is known as one of  
258 the main factors determining the capacity of a species to thrive in an urban environment (41). The DCX  
259 gene is related to neuronal plasticity (56) and experimental approaches revealed that artificial light at  
260 night induces an overexpression of this gene linked to a change in behaviour and expression of  
261 depressive-like behaviour in crows (57). Finally, the CHRNA1 gene is associated with aggressive  
262 behaviour in chicken (58), and higher aggressiveness is commonly observed and hypothesized as  
263 adaptive in urban bird populations (59). Besides, the gene ontology enrichment analysis, performed on  
264 the entire set of genes identified via the outlier genome scan, reinforced these findings since multiple  
265 enriched GO terms were associated with the nervous system and stress response as well as hormonal  
266 response. These results are informative on the type of traits involved in avian urban adaptation in cities  
267 and corroborate previous results from (22, 60, 61) suggesting that natural selection repeatedly acted on  
268 neuronal, behavioural and cognitive traits that could contribute to the phenotypic shifts described in  
269 urban great tits (i.e. more aggressive and exploratory birds, with higher breath rate : 62, 63 & Caizergues  
270 *et al. in prep*)

271

272 Contrary to the common prediction that living in cities is likely to influence epigenomes (24,  
273 25), no genome wide pattern of differentiation in methylation between urban and forest great tits was  
274 detected. However, we observed a difference in mean methylation level between birds from Warsaw  
275 and Barcelona on their autosomes, as well as between males and females on the Z chromosome, showing  
276 that methylation differences were identifiable. In addition, we found no mean difference in methylation  
277 level between habitats, revealing that urbanization did not strongly affect overall methylation levels in  
278 a specific direction. This overall low differentiated methylation context is perfectly suitable to  
279 investigate more localized zones that could differ in their levels of methylation. Note that the strong  
280 methylation contrast between males and females, particularly on sex chromosomes (Figure 1B), is in  
281 line with previous reports in vertebrates (e.g. Teranishi et al. 2001; Waters et al. 2018) showing that  
282 methylation plays a major role in sex differentiation via regulation of gene expression and genetic  
283 imprinting.

284 Despite the non-significant effect of habitat on overall methylation levels, we found a large  
285 number of DMRs between pairs of forest and urban populations, suggesting that urbanization did affect  
286 particular regions of the genome. DMR were less likely to occur within a gene body than by chance, but  
287 it was not the case for promoter or TSS regions. This latter result contrasts with Watson and collaborators  
288 (2020) who recently found an under-representation of DMR in both gene body and regulatory regions  
289 in urban great tits from Malmö (Sweden). Across the three cities, 35.3% of DMR were directly localized  
290 in gene bodies, and 47.3% in TSS or promoter regions, suggesting a potential role in gene expression  
291 modulation. Direction of methylation patterns did not follow any absolute pattern (no over-  
292 representation of hypo- or hypermethylated DMR in urban birds, Figure 3), in line with Watson and  
293 collaborators' analyses on blood sample. However, birds in Barcelona presented significantly more  
294 hypomethylated DMR than hypermethylated ones, a result that warrants further investigations.

295 Only a limited number of urbanization-linked DMR were shared between two or more locations.  
296 In contrast, three times more sex linked DMRs we found in two locations or more. This comparison  
297 suggests that urbanization-linked epigenetic modifications most probably do not occur in a parallel way  
298 across cities, but rather that each city might have its particular epigenetic response. Indeed, in the study  
299 field of urban evolutionary biology, cities are often regarded as valuable replicates of human induced

300 habitats (31, 66), and it is often expected that parallel adaptive responses to similar selective pressures  
301 will occur. This expectation is particularly strong when phenotypes show parallel changes, as is the case  
302 for the Great tit, which is consistently smaller and lays earlier and smaller broods in the various cities  
303 where it has been studied, compared to forest habitats (e.g. 67, 68). However, as discussed above,  
304 parallel adaptation to similar environmental conditions should not be expected in the case of independent  
305 evolution, especially for multilocus traits. Additionally, cities are different from each other because of  
306 a wide array of climatic, cultural, historical and socioeconomic factors (15). In fact, besides the obvious  
307 differences in cities' climatic conditions depending on their position on the globe, land use,  
308 fragmentation and pollutants levels can also largely vary across cities (69). In a general way, pollutants  
309 are known to affect DNA methylation and result in both hypo and hypermethylation, but the patterns of  
310 change observed largely rely on the pollutant involved (70). In this case, differences in cohorts of  
311 pollutants present in cities could be responsible for differences in patterns of methylations. Taken  
312 together, these results highlight the importance of questioning the assumption that cities are replicated  
313 environments that can be considered similar.

314         As mentioned earlier, increasing evidence suggests that DNA methylation can be associated  
315 with environmental and stress factors (env: (12), stress: (71)) especially during early development (72).  
316 Here, we found four genes (POMC, ADAMTS3, PAPD4 et GCC1), associated with DMR that were  
317 previously described in great tits as undergoing major changes in methylation levels in case of exposure  
318 to higher levels of pollutants (73). Notably, the functions of these genes remain to be determined, and  
319 they could thus be interesting to target in future studies. In addition, the past literature has repeatedly  
320 found SERT and DRD4 as two major genes involved in urban specific avian human avoidance (or  
321 wariness) behaviours (see for example Riyahi *et al.* (23) in the Great tit, Garroway and Sheldon (74) in  
322 the blackbird, Van Dongen *et al.* (75) in the black swan & Mueller *et al.* (76) in the burrowing owl) (see  
323 SI Figure S8 to S12 to see patterns of methylation associated with 6 classical great tit linked candidate  
324 genes). In this study, while urban great tits show higher levels of aggressiveness in at least two of the  
325 cities ((43) & Caizergues *et al. in prep*) we found no DMR associated with these two genes in either of  
326 the three forest-city pairs. However, we found a significant urban-related change in methylation linked  
327 to the DRD3 gene, belonging to the same gene family as DRD4 and known to be similarly involved in

328 chicken aggressive behaviour (77). In line with these results, GO analyses revealed enrichment in genes  
329 associated with neuronal functions, behaviour, but also blood, immune and endocrine systems (Table  
330 S9, Figure S3), revealing the potential need of physiological adjustments in urban habitats. Surprisingly,  
331 a recent study on great tit differences of methylation between city and forest habitats in another European  
332 city found no GO enrichment in blood, while some in liver tissue (Watson et al. 2020, note that they  
333 investigated DMSs, Differentially Methylated Sites, which differs from DMRs identified here). These  
334 contrasted results highlight the fact that methylation patterns highly depend on the analysed tissues (11),  
335 and show, once more, that urban linked methylation might not be similar from one city to another. In  
336 addition, it has been demonstrated that DNA methylation can undergo seasonal variation (78). Hence,  
337 analyses on multiple tissues and life-stages replicated in multiple pairs of urban and forest populations  
338 will allow to draw a broader view of the impact of urbanization on global methylation patterns and to  
339 understand replicated parallel occurrence across cities. However, tissue-specific and age-specific  
340 analyses in multiple individuals across several pairs of urban and forest environments poses major  
341 technical, budget and ethical limitations and should be coordinated very carefully. Additionally, specific  
342 drivers of shifts in methylation remain to be disentangled to understand which environmental factors are  
343 responsible for which change in methylation. To do so, experimental settings manipulating  
344 environmental factors such as performed by Mäkinen and collaborators (2019) would be particularly  
345 useful. More integratively, information on how shifts in methylation patterns affect phenotype, fitness,  
346 and adaptation, often remain elusive (79). To our knowledge, a limited number of studies attempted to  
347 link methylation and expression levels in natural population contexts (Derks et al. 2016; Laine et al.  
348 2016), and even fewer in urban habitats (but see e.g. McNew et al. 2017; Watson et al. 2020). Hence,  
349 future work might need to tackle the question of the origin and adaptive significance of these variations  
350 in a controlled framework.

## 351 CONCLUSION

352

353 In this study, we found genomic footprints of selection and differentially methylated regions  
354 associated with urbanization, suggesting that genomic as well as epigenetic processes could play an

355 important role in the rapid adaptation to the urban habitat. To our knowledge, our study is the first to  
356 use replicated pairs of cities and forest habitats when studying urban linked methylation, offering a great  
357 opportunity to investigate convergent responses to anthropogenic environmental conditions. Taken  
358 together, our results revealed limited parallelism between cities regarding selection as well as  
359 methylation patterns, suggesting that cities might not present exactly similar environmental conditions  
360 or that different genetic pathways are involved in adaptation to urban environmental conditions, while  
361 still associated with similar biological functions. Furthermore, we highlight the need to unravel both  
362 environmental origins and evolutionary implications of methylation shifts, to understand to which extent  
363 environmental induced methylation can contribute to adaptation.

364

365

## 366 **MATERIAL AND METHODS**

### 367 **Study sites and sampling**

368 Three pairs of great tit populations in urban and forest environments were sampled in the three  
369 European cities of Barcelona (Spain), Montpellier (France) and Warsaw (Poland). For each location, 10  
370 individuals were sampled within the city and 10 individuals were sampled from nearby forest. Blood  
371 samples were collected from breeding individuals during spring between 2016 and 2018 (except 2  
372 individuals for Barcelona city collected in 2014 and 2015) and kept in alcohol or queen's buffer.  
373 Samples had balanced sex ratio (5 males and 5 females for each population) except for the forest  
374 population of Barcelona where 6 females and 4 males were sampled.

### 375 **DNA extraction, RAD-seq and Reduced-Representation Bisulfite Sequencing**

376 We used QIAGEN DNeasy blood and tissue kit to extract genomic DNA from blood samples  
377 following the provided instructions for nucleated blood samples. DNA was quantified using a NanoDrop  
378 ND8000 spectrophotometer and a Qubit 2.0 fluorometer with the DNA HS assay kit (Life  
379 Technologies). DNA quality was examined on agarose gels. We then performed RAD-sequencing and

380 RRBS-sequencing using standard protocols. For RAD-sequencing (restriction-site-associated DNA  
381 sequencing; Baird et al. 2008), the library preparation was done by the Montpellier GenomiX (MGX)  
382 platform (CNRS, Montpellier), using the enzyme SbfI. Each individual was identified using a unique  
383 six nucleotides tag, individuals were randomly multiplexed in equimolar proportions by libraries of 37  
384 individuals. Each library was sequenced on a lane of an Illumina HiSeq 2500. Paired-end sequencing  
385 was used to produce 150 bp reads. This generated an average of 4.9M reads per individual. The DNA  
386 of 60 individuals were processed twice to test for reliability of the genotyping process. The RRBS-  
387 sequencing started with DNA digestion using MspI restriction enzymes, which cuts CCGG sites and  
388 targets regions that are CG rich, permitting to have a high proportion sequences in promoter regions.  
389 Each individual was identified using a unique six nucleotides tag. Individuals were randomly  
390 multiplexed in equimolar proportions by libraries of 10 individuals. Bisulfite treatment hence converted  
391 unmethylated cytosines into uracil, then converted to thymine after PCR amplification. Each library was  
392 then sequenced on a lane of an Illumina HiSeq 2500. Paired-end sequencing was used to produce 50 bp  
393 reads. This generated an average of 19.3M reads per individual.

#### 394 **Genomic analyses**

395 Fastp v. 0.19.7 (81) was used to trim the RAD-seq reads, keeping reads with a minimum quality  
396 of 15 before mapping individual sequences against the reference genome of the Great tit (Laine et al.  
397 2016, GenBank assembly accession: GCA\_001522545.3) with BWA v0.7.17 (82). Genotyping was  
398 conducted with stacks v2.41 (83) “*gstacks*” and “*population*” functions, using “*snp*” model, filtering for  
399 mapping quality > 10, alpha = 0.05, minor allele frequency > 0.05 and observed heterozygosity  
400 < 0.65. Loci were retained if present in at least 90% of individuals in each population. Loci with extreme  
401 low or high coverage were removed (5% extremes filtered out using vcftools v0.1.15, Danecek et al.  
402 2011). After filtering, 74,137 SNPs were retained for subsequent population genomic analyses.

403 To document genomic variation among urban and rural great tits from the three locations we  
404 used a redundancy analyses (RDA), with location (Barcelona, Montpellier and Warsaw), environment  
405 (urban or rural) and sex as explanatory variables. Partial RDA was also produced to test for each variable

406 effect (environment, location or sex) alone after controlling for all other variables. The effect of a given  
407 factor was considered significant with a p-value < 0.05.

408 To estimate genome-wide differentiation between populations we used Weir and Cockerham's  
409  $F_{ST}$  (37) computed using the StAMPP R package (85). Average  $F_{ST}$  was estimated using all SNPs, and  
410 confidence intervals were assessed using 1000 bootstrap replicates.

411 To identify SNPs potentially under divergent selection between urban and forest habitats we  
412 first used Bayescan v2.1 (38). Analyses were run for each pair of populations (Barcelona, Montpellier  
413 & Warsaw) separately, with default parameter option. As recommended by Foll and Gaggiotti (2008)  
414 we considered SNPs as outliers when they displayed a q-value above the 0.1 threshold.

415 To detect weaker footprints of divergent selection typically expected in polygenic adaptations  
416 in response to complex environmental heterogeneity, a multivariate method was used (39). Following a  
417 similar procedure as described above for the RDA analysis, we used a constrained RDA to investigate  
418 the effect of habitat (forest versus city) and to identify outliers SNPs displaying more than 3 times SD  
419 from the mean score on the constrained axis.

#### 420 **Methylation calling and analyses**

421 The RRBS reads were first trimmed using fastp software v0.19.7 (81), and quality filtered  
422 keeping only reads with a quality > 15. BISMARK software v0.20.0 (86) was used for mapping reads  
423 on the masked reference genome with default parameters and 1 maximum allowed mismatches (-N 1).  
424 Methylation information for cytosine in a CpG context with sufficient coverage ( $\geq 10x$ ) was extracted.  
425 An anova on mean individual methylation was run to test for location, habitat and sex effect on overall  
426 methylation levels.

427 Similarly, to genetic differentiation, an RDA was performed to describe epigenomic variation  
428 in function of location, habitat and sex. A partial RDA was also conducted to test for the habitat effect  
429 alone.



430 To identify differentially methylated regions (DMR) logistic regression including sex as  
431 covariate was performed after tiling regions of 1000pb for each location using MethylKit R package  
432 “*calculateDiffMeth*” function. Only DMR having > 10% overall difference between forest and urban  
433 habitat and q-value < 0.001 were kept. We then investigated if DMRs overlapped genes in 5 kb upstream  
434 and downstream regions using bedtools.

#### 435 **Genes associated to genomic outliers and DMRs, and gene ontology analyses**

436 We investigated if genomic outliers and DMR overlapped genes in 5 kb upstream and  
437 downstream regions using BEDTools v2.28.0 (87). Gene ontology analyses were performed with the R  
438 package topGO (40) to identify potential statistically enriched to investigate if on the list of all genes  
439 associated to RDA genomic outliers and the list of all genes associated to DMR.

440

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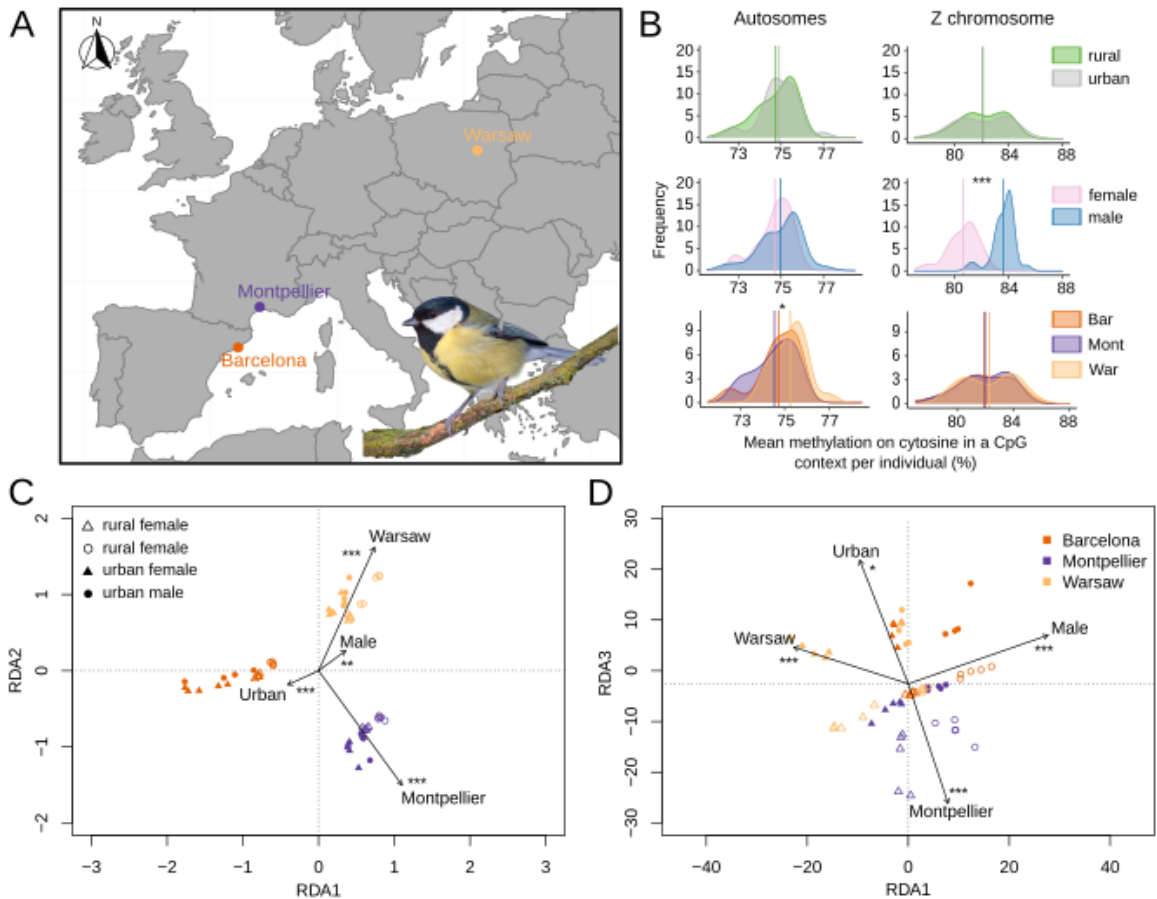
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**FIGURES**

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645

646 *Figure 1: (A) Great tit blood sample locations in Europe (in urban and forest sites in and near*

647 *Barcelona, Montpellier & Warsaw). (B) Distribution of mean percent of methylation on autosomes and*

648 *on the Z chromosome, compared between habitats (forest versus urban), sexes and locations. (C)*

649 *Redundancy analysis (RDA) on genomic data (74,137 filtered SNPs). (D) RDA on methylation levels*

650 *(based on methylation levels observed at 157,741 positions). In (C) and (D), triangles represent rural*

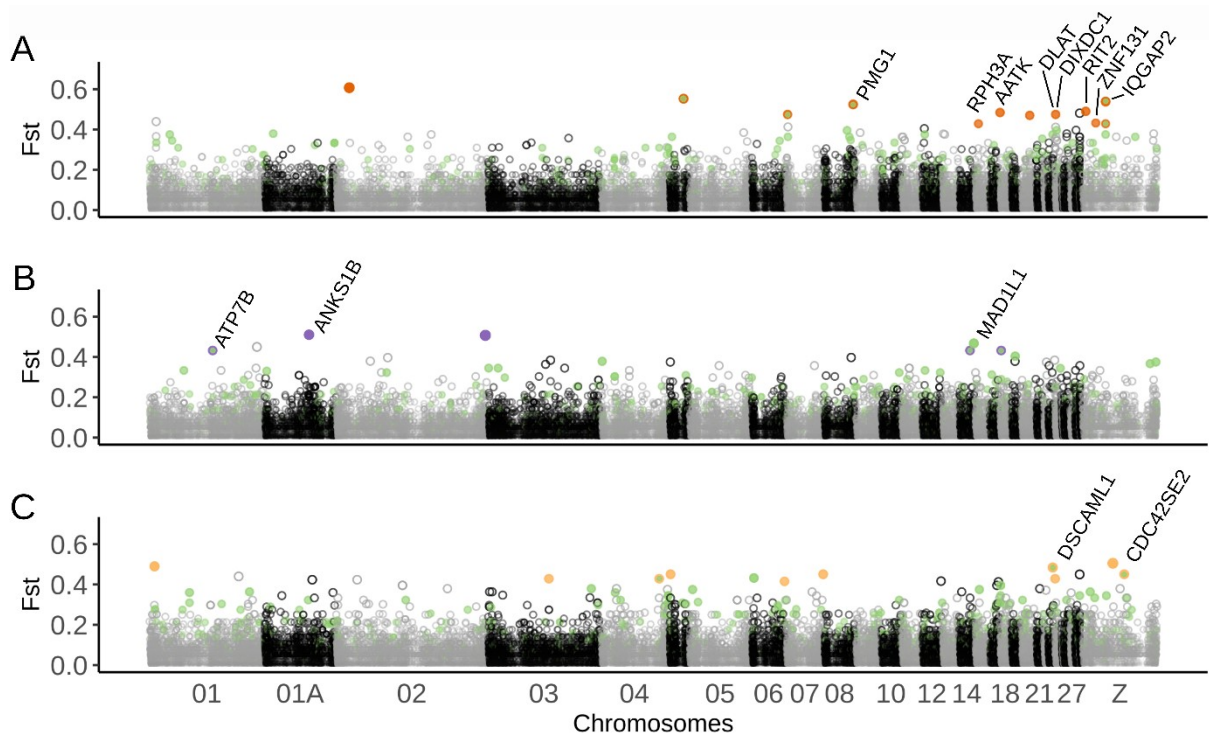
651 *habitats, circles represent urban habitats, empty and solid symbols represent females and males*

652 *respectively. \*\*\* P-value < 0.001, \*\* P-value < 0.01 and \* P-value < 0.05, related to the explanatory*

653 *factors.*

654

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656

657 *Figure 2: Manhattan plot of mean  $F_{ST}$  between urban and forest populations along the Great tit genome*

658 *for (A) Barcelona, (B) Montpellier and (C) Warsaw. Dark orange (A), purple (B) and light orange points*

659 *(C) represent significant outlier SNPs identified by the  $F_{ST}$ -outlier test Bayescan for each population*

660 *pair, given with their associated genes in 5 kb. Green points represent outliers found with the*

661 *multivariate RDA approach. A few SNPs were identified by both the Bayescan and the RDA methods*

662 *and signalled as a green point circled with the colour used for the considered pair.*

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667 *Figure 3: Circos plot of differentially methylated regions (DMRs) identified between populations of*  
 668 *forest and urban great tits in and near Barcelona, Montpellier and Warsaw (from inner to outer circles).*

669 *Red points show hypermethylated regions in urban great tits relative to forest birds, and blue points*

670 *show hypomethylated regions. For graphical clarity, only a subset of genes are represented: genes*

671 *associated with the 10% most extreme DMR (triangles) and genes found associated with DMR in at*

672 *least two cities (stars). Names of the genes found within 5 kb of the represented DMRs are given.*

673