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1 **Morphological stability of rural populations supports their use as controls in urban**
2 **ecology studies**

3
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11 Running title: Morphological stability of rural populations

12
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23 **ABSTRACT**

24 The expansion of urban environments and how animals may be affected by them are
25 being increasingly investigated, leading to a surge in urban ecology studies. Many urban
26 ecology studies involve a direct comparison between rural and urban populations, or the use of
27 urban gradients along a continuum from rural to urban areas. The implicit, although not properly
28 investigated, assumption in these rural vs urban comparisons is that the rural populations offer a
29 control that represents a lack of the anthropogenic stressors affecting the urban populations.
30 Here we used museum skulls from 14 rodent species to conduct two separate studies,
31 measuring fluctuating asymmetry (FA) as a proxy of developmental stress to assess the effect
32 of anthropogenic disturbance. First, we compared urban and rural specimens of *Mus musculus*
33 (house mouse) to validate our methodological approach. Second, we compared rural specimens
34 from 14 rodent species collected during the last two centuries across Austria. We hypothesised
35 that FA in rural populations has not increased over the last two centuries, which would support
36 the use of rural populations as a proper control in rural vs urban comparisons. We found higher
37 morphological asymmetry in urban populations of *Mus musculus* compared to rural populations,
38 which is consistent with similar studies in other species. However, we did not find any significant
39 increase in FA over time in rural populations for any of the studied species. This offers some
40 support to the common practice of using rural populations as a control in rural vs urban
41 comparisons when assessing the effects of urbanisation.

42 **Keywords: Anthropocene; fluctuating asymmetry; rodents; skulls; urban ecology; urban**
43 **vs rural comparisons**

44 INTRODUCTION

45 The exponential growth of the human population and the increased percentage of humans
46 moving into urbanised areas has led to a sustained expansion of urban environments (United
47 Nations Population Division, 2018). This has entailed the emergence of new cities and the
48 expansion of existing cities, but it has also led to an increase in many anthropogenic activities in
49 the rural areas surrounding cities (Grimm *et al.* 2008; McKinney 2002). Urban animals are
50 affected by several anthropogenic stressors and respond by adjusting their behaviour,
51 morphology and physiology, some of these responses involving evolutionary changes (Johnson
52 & Munshi-South 2017; Ouyang *et al.* 2018; Rivkin *et al.* 2019; Szulkin *et al.* 2020). Given the
53 relevance of the effects of urbanisation on the survival of a growing number of species, and the
54 quality of habitats and ecosystem services, there has been a surge in urban ecology studies
55 during the last 30 years (Magle *et al.* 2012; McDonnell 2011; Ouyang *et al.* 2018). The majority
56 of urban ecology studies have involved a direct comparison between rural and urban
57 populations, or studying urban gradients along a continuum extending from highly urbanised
58 areas to rural areas.

59 The implicit assumption with rural vs urban comparisons is that the rural populations offer
60 a control that represents a lack of the anthropogenic stressors affecting the urban populations.
61 However, the reach of human influence in non-urban habitats is pervasive, particularly in the
62 rural areas adjacent to cities, including increases in intensive agriculture; use of fertilizers and
63 pesticides; habitat deforestation, fragmentation, and degradation; and road networks (Coda *et*
64 *al.* 2016; Geiger *et al.* 2010; Marchand *et al.* 2003; Tschardtke *et al.* 2005). Anthropogenic
65 disturbance in rural areas alters the distribution and abundance of native species and
66 communities, which exacerbates the impacts that habitat disturbances have on biodiversity
67 (Ewers & Didham 2006; Geiger *et al.* 2010). However, it is unclear whether such anthropogenic
68 disturbances significantly impact the phenotype of rural animals (Coda *et al.* 2016).

69 The assumption that rural populations can be an appropriate control to assess the effect
70 of urbanisation on urban populations has not been properly investigated, despite being a
71 cornerstone in the emerging field of urban ecology. However, if rural populations have also been
72 affected by similar anthropogenic stressors impacting urban populations (e.g. noise, light, and
73 chemical pollution), rural vs urban comparisons may grossly underestimate the effects of
74 urbanisation in urban populations.

75 Investigating the effects of anthropogenic stressors in rural populations is
76 methodologically complicated by the lack of an appropriate control. Some studies have
77 considered different types of rural conditions, e.g. organic vs conventional farming (Coda *et al.*
78 2016), but these studies cannot address whether the effects of anthropogenic disturbance on
79 rural populations is minimal compared to the level of disturbance in urban environments.
80 Instead, an effective approach is to investigate rural populations in a given region over a wide
81 time period, including times before and after the accelerated globalisation and environment-
82 altering activities that were launched around the middle of the 20th century (Pergams & Lawler
83 2009). One option to implement this approach is to use museum specimens from species with a
84 representative time series (Pergams & Lawler 2009; Schmitt *et al.* 2018).

85 Morphological features in museum specimens can be used to assess the effect of
86 environmental stressors in rural populations over time. In this context, measuring developmental
87 instability is particularly fitting (Leamy & Klingenberg 2005). Developmental instability is the
88 inability of an organism to produce a perfectly symmetrical phenotype because of environmental
89 stressors affecting its normal development (Dongen 2006; Leamy & Klingenberg 2005). A
90 frequently-used measure of developmental instability is fluctuating asymmetry (FA), defined as
91 small, random deviations from perfect symmetry in a population (Leamy & Klingenberg 2005;
92 Palmer & Strobeck 1986). FA is calculated by subtracting the value of a morphological feature
93 on the right side of the body from the value on the left side of the body (Palmer 1994). These FA
94 values will be negative in some individuals and positive in others, but they should not differ from

95 zero at the population level (Dongen 2006; Van Valen 1962). Higher FA values indicate a higher
96 incidence of environmental stressors affecting the normal development of organisms (Beasley
97 *et al.* 2013). Consequently, FA of morphological traits has been widely used as an indicator of
98 environmental and/or genetic stress driven by many different factors, e.g. inbreeding, extreme
99 temperatures, food deprivation, parasitism, habitat fragmentation, or pollution (Beasley *et al.*
100 2013; De Coster *et al.* 2013; Lazić *et al.* 2013; Nunes *et al.* 2001). Importantly, FA has been
101 used to study both the effect of urbanisation (Lazić *et al.* 2013; Winchell *et al.* 2019) and the
102 effects of anthropogenic disturbance in rural areas (Coda *et al.* 2016; Marchand *et al.* 2003).

103 We used rodent skulls to conduct two separate studies. First, as a proof-of-concept we
104 compared house mice (*Mus musculus*) from urban and rural regions in and around the city of
105 Vienna since 1980. We predicted higher FA in urban mice than in rural mice. This study was
106 intended to provide a baseline for the effect of urbanisation, to better interpret any changes over
107 time in rural populations. Second, we determined if FA in 14 rodent species has increased in
108 rural areas over many decades. Investigating several species is important, because changes in
109 FA may be species-specific and driven by different ecological requirements (Coda *et al.* 2016).
110 An increase in FA over time would indicate that rural populations have been noticeably affected
111 by anthropogenic activity and that any rural vs urban comparisons may underestimate the effect
112 of urbanisation. In contrast, a lack of changes in FA over time would support the common use of
113 rural populations as a control in rural vs urban comparisons in urban ecology studies.

114

115 **METHODS**

116 We used skulls from specimens stored in the Mammal Collection at the Natural History
117 Museum Vienna (Naturhistorisches Museum Wien) to conduct two separate studies, one
118 comparing urban and rural specimens of *Mus musculus*, and another one investigating rural
119 specimens from several species across a long timeframe (1824 to 2014).

120 For the urban vs rural study, we selected 61 adult specimens of *Mus musculus* that were
121 collected in the city of Vienna or in rural sites nearby Vienna since 1980 (N = 23 from rural sites,
122 and N = 38 from urban sites). Each specimen was classified as urban or rural using the
123 available location information associated to the specimen (see Supplementary dataset 1 for sex
124 and location information). For the second study, we selected adult specimens (juvenile or adult
125 status was based on information available in the museum records) that were collected in rural
126 areas in Austria (see location details in Supplementary dataset 2). If many specimens of a
127 species were available for a given year, we randomly chose a subsample of intact skulls with a
128 similar representation of males and females, in order to obtain a representative coverage of
129 collection years for that species. Across all species, specimens were collected from 1824 to
130 2014 (maximum range was 1824 to 2006 for *Arvicola terrestris*, and minimum range was 1927
131 to 1998 for *Muscardinus avellanarius*). In total, we selected 495 specimens from 14 species: 39
132 *Apodemus flavicollis*; 46 *Apodemus sylvaticus*; 38 *Arvicola terrestris*; 35 *Cricetus cricetus*; 29
133 *Glis glis*; 24 *Micromys minutus*; 48 *Microtus arvalis*; 33 *Microtus subterraneus*; 16 *Muscardinus*
134 *avellanarius*; 52 *Mus musculus*; 36 *Myodes glareolus*; 35 *Rattus norvegicus*; 27 *Sciurus*
135 *vulgaris*; and 37 *Spermophilus citellus* (see Supplementary dataset 2).

136 We used a Sony A7RII camera mounted on a tripod, with a macro lens Sony FE 90mm
137 F2.8 Macro G OSS, to take all photographs. We took two photographs per skull, one dorsal, and
138 one ventral. We placed a ruler next to the specimen and at the same level as the top surface of
139 the skull. Spirit levels were used on the camera and the ruler to align them with the specimen.

140 Photographs were analysed using ImageJ 1.51s (Schneider *et al.* 2012). We used the
141 ruler photographed next to the skull to set up the scale. We measured the total length of the
142 skull, from the anterior intersection of the nasal bones to the posterior end of the skull (from
143 point 1 to 2 in Fig. 1A, respectively). We also measured four dorsal and four ventral lengths in
144 both the right and left sides of the skull. The four dorsal lengths were: BZA (Bregma-Zygomatic
145 Arch), from bregma (intersection of frontal bones and parietal bones at midline; point 3 in Fig.

146 1A) to the external point in the intersection of bones in the zygomatic arch (point 4 in Fig. 1A);
147 NASAL, from the anterior intersection of the nasal bones to the most anterior point of the
148 zygomatic arch (from point 1 to 5 in Fig. 1A, respectively); NAZ (NASal-Zygomatic), from the
149 caudal point in the intersection of nasal bones (point 6 in Fig. 1A) to the rostral end of the
150 zygomatic arch (point 5 in Fig. 1A); and POSDOR (POSTerior and DORSal), from the posterior
151 end of the skull to the posterior end of the orbital space (from point 2 to 7 in Fig. 1A,
152 respectively). The four ventral lengths were: CENVEN (CENTral and VENtral), from the
153 posteriormost point of the suture between palatines and the anterior border of the
154 mesopterygoid fossa (point 8 in Fig. 1B) to the posteriormost point of the incisive foramen (point
155 9 in Fig. 1B); FORAMEN, from the posteriormost point of the incisive foramen to the
156 anteriormost point of the incisive foramen (from point 9 to 10 in Fig. 1B, respectively); MIDVEN
157 (MIDsection and VENtral), from the posteriormost point of the suture between palatines and the
158 anterior border of the mesopterygoid fossa (point 8 in Fig. 1B) to the posteriormost point of the
159 orbital space (point 11 in Fig. 1B); and POSVEN (POSTerior and VENtral), from the anteriormost
160 point of the foramen magnum (point 12 in Fig. 1B) to the posteriormost point of the orbital space
161 (point 11 in Fig. 1B).

162 Some skulls were partially damaged, so not all variables were measured for each
163 specimen (as indicated by NA in the Supplementary datasets). Each measurement was taken
164 twice with an interval of at least one month between the first and the second measure. If the two
165 measures of the same length differed by 1% or more, two new measures were taken and this
166 was repeated until both measures differed by less than 1%. To further minimise sampling error,
167 all skull measures were taken by the same person (TR).

168

169 ***Statistical Analysis***

170 We used R version 3.5.3 (R Core Team 2019) for all analyses. To check for the existence
171 of FA and directional asymmetry (DA) for each one of the variables, we used mixed models

172 (Palmer 1994). DA is an asymmetry that consistently favours one side of the body over the
173 other, e.g. the location of the mammalian heart (Van Valen 1962). We log-transformed all
174 measurements and fit a mixed model for each variable and for each species, using the function
175 lmer (package lmerTest). In these models, we included side (right or left side of the skull) as a
176 fixed effect, the interaction between side and individual, and individual as a random effect. A
177 statistically significant difference between the right and left side across individuals would
178 indicate DA. A statistically significant interaction between side and individual would indicate that
179 the measurement for the right side was larger than the left side for some individuals but the
180 opposite was true for other individuals, i.e. an indication of FA for that variable. Variables with
181 significant levels of DA can confound the estimation of real FA and it is recommended to avoid
182 any such variables from FA analyses (Dongen 2006; Palmer 1986, 1994). For the first study
183 (comparing rural vs urban specimens), we detected significant DA for the variables BZA,
184 NASAL, and POSDOR, so we did not include these variables in the main analyses. For the
185 second study (rural specimens from several species over time), we detected significant DA for
186 the variables NAZ, BZA, MIDVEN, and FORAMEN for several species, so we did not include
187 these variables in the main analyses. Therefore, we considered the following morphological
188 variables in the main analyses: NASAL, POSDOR, POSVEN, and CENVEN.

189 To assess FA in the main analyses, we first calculated a FA index for each individual and
190 for each variable. In each case, we considered 4 values, i.e. the two replicated measures for
191 each side. First, we calculated the mean for each side using the two replicated measures.
192 Second, we subtracted the left value from the right value and used the absolute value from this
193 subtraction as the FA index. Third, we log-transformed the FA index (three FA values were
194 exactly zero prior to log-transformation so we did not include these values in the analyses;
195 adding a constant to all values prior to log-transformation to retain the aforementioned three FA
196 values did not affect any results). We also calculated the mean skull length using the two
197 replicated values for each individual. The datasets used in the analyses are provided as

198 Supplementary material. For each variable we implemented a linear model using the *lm*
199 function. In the first-study (urban vs rural *Mus musculus*) analyses, the FA values were the
200 response, and we included as explanatory variables the type of habitat (urban or rural), sex, and
201 year of capture (continuous variable). We also included skull length as an offset. In the second-
202 study (assessing FA values of several species over time in rural locations) analyses, the FA
203 values were the response, the explanatory variables were sex, year of capture, species and the
204 interaction between species and year of capture, and skull length was an offset. Following
205 Crawley (2002), we started with a full model and excluded terms sequentially until the model
206 contained only statistically significant terms.

207

208 **RESULTS**

209 In the rural vs urban study, the five dependent variables (CENVEN, FORAMEN, MIDVEN,
210 NAZ, and POSVEN) did not differ between males and females ($p > 0.05$ for all analyses). We
211 did not detect any significant effect of habitat type for the variables CENVEN, FORAMEN, NAZ
212 and POSVEN ($p > 0.05$ for all analyses); however, FA values for the variable MIDVEN were
213 significantly higher in urban specimens than in rural specimens ($F_{1,52} = 11.26$, $p = 0.0015$; Fig.
214 2).

215 In the study considering rural specimens over a large period of time, we found species-
216 specific differences in FA for the four considered morphological measurements (CENVEN: $p <$
217 0.0001 ; NASAL: $p = 0.028$; POSDOR: $p = 0.028$; and POSVEN: $p = 0.004$). However, pairwise
218 comparisons between species (using the Tukey method for the adjustment of p values with the
219 function *emmeans* from the package *emmeans*) were only statistically significant for CENVEN,
220 with FA values from *Arvicola terrestris* being significantly higher than those from *Apodemus*
221 *sylvaticus*, *Apodemus flavicolis*, *Mus musculus*, *Micromys minutus*, *Microtus subterraneus*,
222 *Microtus arvalis*, *Myodes glareolus* and *Spermophilus citellus* ($p < 0.05$ for all analyses), and FA

223 values from *Cricetus cricetus* being significantly higher than those from *Apodemus flavicollis*,
224 *Micromys minutus*, *Microtus subterraneus* and *Myodes glareolus* ($p < 0.05$ for all analyses). Sex
225 and the interaction between species and year of capture did not affect FA values for any of the
226 morphological measurements ($p > 0.05$ for all analyses). Importantly, FA values did not differ by
227 year of capture in the case of 3 morphological measurements (CENVEN, NASAL, and
228 POSVEN: $p > 0.05$ for all analyses), and FA values for POSDOR significantly decreased over
229 time ($F_{1,423} = 10.59$, $p = 0.0012$; Fig. 3).

230 We also considered the effect of year of capture on FA for each species separately. After
231 adjusting the p values because of multiple comparisons using the Bonferroni correction, we
232 found no significant change in FA over time for any individual species (adjusted p values > 0.05
233 for all analyses).

234

235 **DISCUSSION**

236 We found a higher incidence of FA in the midsection of the skull in urban house mice
237 compared to rural house mice. However, we did not detect an increase in FA in rural
238 populations of several rodent species over the last two centuries. In fact, the only variable that
239 differed over time was POSDOR (involving the posterior section of the skull), but contrary to our
240 hypothesis this variable declined over time when all species were included in the model.

241 Several studies have found increased levels of asymmetry in response to different types
242 of anthropogenic disturbance, such as high levels of urbanisation, in many organisms, including
243 plants, invertebrates and vertebrates (Banaszak-Cibicka *et al.* 2018; Cuevas-Reyes *et al.* 2013;
244 Eeva *et al.* 2000; Elek *et al.* 2014; Lazić *et al.* 2013; Teixeira *et al.* 2006; Weller & Ganzhorn
245 2004). Our finding of higher asymmetry in the skulls of urban house mice in Vienna compared to
246 rural specimens adds to this literature, although only one of five measured variables, involving
247 the middle section of the skull, differed between urban and rural populations. It must be noted

248 however that there are some conflicting results in studies using FA to assess the effect of urban
249 stressors. For example, in carabid beetles, FA increased with urbanisation in species
250 considered to be negatively affected by urbanisation, whereas such a relationship was not found
251 in species that are more tolerant to urbanisation (Weller & Ganzhorn 2004); however, in another
252 study FA did not change along rural-urban gradients (Elek *et al.* 2014). Similarly, different
253 studies in lizards have reported an increase in FA in response to urbanisation (Lazić *et al.*
254 2013), no changes in FA in response to urbanisation (Sacchi *et al.* 2018), and even a decrease
255 in FA in response to urbanisation, the authors interpreting this last result as natural selection
256 being stronger in urban populations and asymmetrical individuals being less likely to survive to
257 adulthood (Winchell *et al.* 2019).

258 Our results on rural populations can be interpreted as rural animals from some species not
259 being particularly affected by the levels of anthropogenic disturbance that have taken place in
260 rural landscapes over the last decades (O'Donnell & delBarco-Trillo 2020). It must be noted
261 however that our study was not intended to assess how fitness of rural animals may have
262 changed over the decades in response to human activity, nor what are the different factors from
263 such human activity more directly responsible for any potential effects on fitness. Our data
264 cannot possibly address these questions nor contribute to the controversial relationship
265 between symmetry and fitness (Dongen 2006; Lens *et al.* 2002; Møller 1997). More detailed
266 studies may provide more nuanced results (Sacchi *et al.* 2018). For example, FA was higher in
267 bank voles (*Myodes glareolus*) in disturbed rural areas in the northern coast of France than in
268 undisturbed rural areas (Marchand *et al.* 2003).

269 Traits that have a direct impact on survival or fitness, e.g. extremities and traits involved in
270 foraging, are under strong selection and may be developmentally more stable (De Coster *et al.*
271 2013) and thus not reflect the effects of environmental impacts particularly well (Clarke 1995;
272 Dongen 2006; Winchell *et al.* 2019). In contrast, traits for which some level of asymmetry may
273 not necessarily result in a decrease in fitness may be better indicators of the environmental

274 stress experienced by individuals during development (Clarke 1995). Although it is unclear
275 whether small skull asymmetries may directly decrease fitness, there are nonetheless several
276 studies reporting that poor environmental conditions increase skull asymmetry (Maestri *et al.*
277 2015; Marchand *et al.* 2003; Oleksyk *et al.* 2004; Zakharov & Yablokov 1990).

278 In our study, the lack of an increase in FA over the last decades in rural areas was
279 consistent across different species with different ecological requirements and thus possibly
280 affected differently by any given anthropogenic activity. For example, rodent species closely
281 associated to bodies of water (e.g. *Arvicola terrestris*, the European water vole) may be more
282 affected by fertilisers and other contaminants reaching and accumulating in those water bodies.
283 We selected a broad array of species with different ecological requirements, including
284 commensal species (*Mus musculus*, house mouse; *Rattus norvegicus*, brown rat; and
285 *Apodemus sylvaticus*, wood mouse), species associated to water bodies (e.g. *Arvicola terrestris*
286 and *Rattus norvegicus*), species associated with grasslands (e.g. *Micromys minutus*, Eurasian
287 harvest mouse; and *Microtus arvalis*, common vole), species mainly associated to woodlands
288 (e.g. *Myodes glareolus*; *Apodemus flavicollis*, yellow-necked mouse; *Glis glis*, edible dormouse;
289 *Muscardinus avellanarius*, hazel dormouse; and *Sciurus vulgaris*, Eurasian red squirrel),
290 species restricted to low-land areas where they are likely to be exposed to diverse
291 anthropogenic activities (e.g. *Cricetus cricetus*, the common or European hamster), or species
292 restricted to areas with soft turf where they can dig burrow systems (*Spermophilus citellus*,
293 European ground squirrel). Despite these ecological differences, we did not find any increase in
294 FA over time in any individual species. We found, however, that two species (*Arvicola terrestris*
295 and *Cricetus cricetus*) had significantly higher FA than many other species, regardless of year of
296 capture. These two species can be closely exposed to pollution (e.g. pesticides) given their
297 habitat preferences, but so are other species included in our analyses, so it is unclear why FA
298 values were higher in these particular species. Similarly, the species differences that we

299 observed cannot be explained through a distinction between specialist and generalist species
300 (Coda *et al.* 2016; Teixeira *et al.* 2006).

301 There are several measures of stress that can be used by ecologists to understand any
302 impacts of anthropogenic activities on wildlife, and the use of FA is only one of them. Other
303 indices of stress in wildlife includes measures of oxidative stress and antioxidant responses
304 (Chatelain *et al.* 2020), measures of stress hormones (Beauguard *et al.* 2019; Iglesias-Carrasco
305 *et al.* 2020), and immune responses (Ibáñez-Álamo *et al.* 2020). All these different measures
306 inform about stress conditions acting upon different developmental stages and their combined
307 study can help us to dilucidate how anthropogenic factors affect different populations, e.g. rural
308 and urban populations (Isaksson 2020).

309 Our study emphasises the importance of museum collections to address questions that
310 may not be easily tackled otherwise. For example, by using museum specimens collected over
311 the last centuries we can investigate how populations respond over time to changing
312 environmental conditions, e.g. the impact of anthropogenic disturbance (Pergams & Lacy 2008;
313 Pergams & Lawler 2009; Snell-Rood & Wick 2013). We also want to highlight the importance of
314 increasing the representation of urban populations in museum collections to facilitate the
315 implementation of future studies that are not limited by low sample sizes, discontinuous time
316 scales, or an unbalanced presence of specimens collected across gradients from highly urban
317 to highly non-urban locations.

318 In conclusion, our study offers some validation to the use of rural populations as an
319 appropriate control when investigating the effect of urbanisation on urban populations, with the
320 caveats that this may not be the case for all species, and that there may be abiotic and
321 ecological factors uniquely affecting rural habitats. Several measures, e.g. FA, measures of
322 stress, and toxin exposure, can be compared in future studies to justify the use of rural
323 populations as the baseline for the impacts of urbanisation. Ultimately, rural habitats cannot be
324 defined by the absence of human disturbance, and any comparison of habitats should quantify

325 the factors affecting each habitat and the impact that those factors may have on the populations
326 under study.

327

328 **DECLARATIONS**

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331 **Ethics approval.** Not applicable.

332 **Conflicts of interest.** The authors declare no conflict of interest.

333 **Consent to participate.** Not applicable.

334 **Consent for publication.** Not applicable.

335 **Availability of data and material.** Datasets are provided as Supplementary material.

336 **Code availability.** R scripts are available from the corresponding author.

337 **Authors' contributions.** JdB-T conceived the ideas and designed the methodology; TR
338 collected the data; JdB-T analysed the data; TR and JdB-T led the writing of the manuscript. All
339 authors contributed critically to the drafts and gave final approval for publication.

340

341 **REFERENCES**

342 Banaszak-Cibicka, W., Fliszkiewicz, M., Langowska, A., Zmihorski, M. (2018) Body size and
343 wing asymmetry in bees along an urbanization gradient. *Apidologie* 49, 297-306. doi:
344 10.1007/s13592-017-0554-y

345 Beasley, D. A. E., Bonisoli-Alquati, A., Mousseau, T. A. (2013) The use of fluctuating asymmetry
346 as a measure of environmentally induced developmental instability: A meta-analysis.
347 *Ecological Indicators* 30, 218-226. doi: 10.1016/j.ecolind.2013.02.024

348 Beaugeard, E., Brischoux, F., Henry, P. Y., Parenteau, C., Trouve, C., Angelier, F. (2019) Does
349 urbanization cause stress in wild birds during development? Insights from feather

350 corticosterone levels in juvenile house sparrows (*Passer domesticus*). Ecology and
351 Evolution 9, 640-652. doi: 10.1002/ece3.4788

352 Chatelain, M., Drobniak, S. M., Szulkin, M. (2020) The association between stressors and
353 telomeres in non-human vertebrates: a meta-analysis. Ecol Lett 23, 381-398. doi:
354 10.1111/ele.13426

355 Clarke, G. M. (1995) Relationships between developmental stability and fitness: Application for
356 conservation biology. Conservation Biology 9, 18-24. doi: 10.1046/j.1523-
357 1739.1995.09010018.x

358 Coda, J., Gomez, D., Martínez, J. J., Steinmann, A., Priotto, J. (2016) The use of fluctuating
359 asymmetry as a measure of farming practice effects in rodents: A species-specific
360 response. Ecological Indicators 70, 269-275. doi: 10.1016/j.ecolind.2016.06.018

361 Crawley, M. J. (2002) Statistical Computing: An Introduction to Data Analysis Using S-Plus.
362 Wiley, Chichester, UK.

363 Cuevas-Reyes, P., Gilberti, L., González-Rodríguez, A., Fernandes, G. W. (2013) Patterns of
364 herbivory and fluctuating asymmetry in *Solanum lycocarpum* St. Hill (Solanaceae) along
365 an urban gradient in Brazil. Ecological Indicators 24, 557-561. doi:
366 10.1016/j.ecolind.2012.08.011

367 De Coster, G., Van Dongen, S., Malaki, P., Muchane, M., Alcántara-Exposito, A., Matheve, H.,
368 Lens, L. (2013) Fluctuating asymmetry and environmental stress: Understanding the role
369 of trait history. Plos One 8, e57966. doi: 10.1371/journal.pone.0057966

370 Dongen, S. V. (2006) Fluctuating asymmetry and developmental instability in evolutionary
371 biology: Past, present and future. Journal of Evolutionary Biology 19, 1727-1743. doi:
372 10.1111/j.1420-9101.2006.01175.x

373 Eeva, T., Tanhuanpää, S., Råbergh, C., Airaksinen, S., Nikinmaa, M., Lehikoinen, E. (2000)
374 Biomarkers and fluctuating asymmetry as indicators of pollution-induced stress in two

375 hole-nesting passerines. *Functional Ecology* 14, 235-243. doi: 10.1046/j.1365-
376 2435.2000.00406.x

377 Elek, Z., Lövei, G. L., Bárti, M. (2014) No increase in fluctuating asymmetry in ground beetles
378 (Carabidae) as urbanisation progresses. *Community Ecology* 15, 131-138. doi:
379 10.1556/comec.15.2014.2.1

380 Ewers, R. M., Didham, R. K. (2006) Confounding factors in the detection of species responses
381 to habitat fragmentation. *Biological Reviews* 81, 117-142. doi:
382 10.1017/s1464793105006949

383 Geiger, F., Bengtsson, J., Berendse, F., Weisser, W. W., Emmerson, M., Morales, M. B.,
384 Ceryngier, P., Liira, J., Tschardtke, T., Winqvist, C., Eggers, S., Bommarco, R., Pärt, T.,
385 Bretagnolle, V., Plantegenest, M., Clement, L. W., Dennis, C., Palmer, C., Oñate, J. J.,
386 Guerrero, I., Hawro, V., Aavik, T., Thies, C., Flohre, A., Hänke, S., Fischer, C., Goedhart,
387 P. W., Inchausti, P. (2010) Persistent negative effects of pesticides on biodiversity and
388 biological control potential on European farmland. *Basic and Applied Ecology* 11, 97-105.
389 doi: 10.1016/j.baae.2009.12.001

390 Grimm, N. B., Faeth, S. H., Golubiewski, N. E., Redman, C. L., Wu, J., Bai, X., Briggs, J. M.
391 (2008) Global change and the ecology of cities. *Science* 319, 756-760. doi:
392 10.1126/science.1150195

393 Ibáñez-Álamo, J. D., Jimeno, B., Gil, D., Thomson, R. L., Aguirre, J. I., Díez-Fernández, A.,
394 Faivre, B., Tieleman, B. I., Figuerola, J. (2020) Physiological stress does not increase with
395 urbanization in European blackbirds: Evidence from hormonal, immunological and cellular
396 indicators. *Sci Total Environ* 721, 137332. doi: 10.1016/j.scitotenv.2020.137332

397 Isaksson, C. (2020) Urban ecophysiology: beyond costs, stress and biomarkers. *Journal of*
398 *Experimental Biology* 223, jeb203794. doi: 10.1242/jeb.203794

399 Iglesias-Carrasco, M., Aich, U., Jennions, M. D., Head, M. L. (2020) Stress in the city: meta-
400 analysis indicates no overall evidence for stress in urban vertebrates. *Proceedings of the*

401 Royal Society of London B: Biological Sciences 287, 20201754. doi:
402 10.1098/rspb.2020.1754

403 Johnson, M. T. J., Munshi-South, J. (2017) Evolution of life in urban environments. *Science* 358,
404 eaam8327. doi: 10.1126/science.aam8327

405 Lazić, M. M., Kaliontzopoulou, A., Carretero, M. A., Crnobrnja-Isailović, J. (2013) Lizards from
406 urban areas are more asymmetric: Using fluctuating asymmetry to evaluate environmental
407 disturbance. *Plos One* 8, e84190. doi: 10.1371/journal.pone.0084190

408 Leamy, L. J., Klingenberg, C. P. (2005) The genetics and evolution of fluctuating asymmetry.
409 *Annual Review of Ecology, Evolution, and Systematics* 36, 1-21. doi:
410 10.1146/annurev.ecolsys.36.102003.152640

411 Lens, L., Van Dongen, S., Kark, S., Matthysen, E. (2002) Fluctuating asymmetry as an indicator
412 of fitness: Can we bridge the gap between studies? *Biological Reviews* 77, 27-38. doi:
413 10.1017/s1464793101005796

414 Maestri, R., Fornel, R., Galiano, D., de Freitas, T. R. O. (2015) Niche suitability affects
415 development: Skull asymmetry increases in less suitable areas. *Plos One* 10, e0122412.
416 doi: 10.1371/journal.pone.0122412

417 Magle, S. B., Hunt, V. M., Vernon, M., Crooks, K. R. (2012) Urban wildlife research: Past,
418 present, and future. *Biological Conservation* 155, 23-32. doi:
419 10.1016/j.biocon.2012.06.018

420 Marchand, H., Paillat, G., Montuire, S., Butet, A. (2003) Fluctuating asymmetry in bank vole
421 populations (Rodentia, Arvicolinae) reflects stress caused by landscape fragmentation in
422 the Mont-Saint-Michel Bay. *Biological Journal of the Linnean Society* 80, 37-44. doi:
423 10.1046/j.1095-8312.2003.00206.x

424 McDonnell, M. J. (2011) The history of urban ecology: An ecologist's perspective. In: J.
425 Niemelä, J. H. Breuste, T. Elmquist, G. Guntenspergen, P. James, N. E. McIntyre (eds)

426 Urban Ecology: Patterns, Processes and Applications. Oxford University Press, Oxford,
427 pp. 5-13

428 McKinney, M. L. (2002) Urbanization, biodiversity, and conservation. *Bioscience* 52, 883-890.

429 Møller, A. P. (1997) Developmental stability and fitness: A review. *American Naturalist* 149, 916-
430 932. doi: doi:10.1086/286030

431 Nunes, A. C., Auffray, J. C., Mathias, M. L. (2001) Developmental instability in a riparian
432 population of the Algerian mouse (*Mus spretus*) associated with a heavy metal-polluted
433 area in central Portugal. *Archives of Environmental Contamination and Toxicology* 41,
434 515-521. doi: 10.1007/s002440010279

435 O'Donnell, K., delBarco-Trillo, J. (2020) Changes in the home range sizes of terrestrial
436 vertebrates in response to urban disturbance: a meta-analysis. *Journal of Urban Ecology*
437 6. doi: 10.1093/jue/juaa014

438 Oleksyk, T. K., Novak, J. M., Purdue, J. R., Gashchak, S. P., Smith, M. H. (2004) High levels of
439 fluctuating asymmetry in populations of *Apodemus flavicollis* from the most contaminated
440 areas in Chernobyl. *Journal of Environmental Radioactivity* 73, 1-20. doi:
441 10.1016/j.jenvrad.2003.07.001

442 Ouyang, J. Q., Isaksson, C., Schmidt, C., Hutton, P., Bonier, F., Dominoni, D. (2018) A new
443 framework for urban ecology: An integration of proximate and ultimate responses to
444 anthropogenic change. *Integrative and Comparative Biology* 58, 915-928. doi:
445 10.1093/icb/icy110

446 Palmer, A. R. (1994) Fluctuating asymmetry analyses: A primer. In: T. A. Markow (ed)
447 *Developmental Instability: Its Origins and Evolutionary Implications*. Kluwer, Dordrecht,
448 Netherlands, pp. 335-364

449 Palmer, A. R., Strobeck, C. (1986) Fluctuating asymmetry: Measurement, analysis, patterns.
450 *Annual Review of Ecology and Systematics* 17, 391-421. doi:
451 10.1146/annurev.es.17.110186.002135

452 Pergams, O. R. W., Lacy, R. C. (2008) Rapid morphological and genetic change in Chicago-
453 area *Peromyscus*. *Molecular Ecology* 17, 450-463. doi: 10.1111/j.1365-
454 294X.2007.03517.x

455 Pergams, O. R. W., Lawler, J. J. (2009) Recent and widespread rapid morphological change in
456 rodents. *Plos One* 4. doi: 10.1371/journal.pone.0006452

457 R Core Team (2019). R: A language and environment for statistical computing. R Foundation for
458 Statistical Computing, Vienna, Austria. ISBN 3-900051-07-0, URL [http://www.R-](http://www.R-project.org)
459 [project.org](http://www.R-project.org)

460 Rivkin, L. R., Santangelo, J. S., Alberti, M., Aronson, M. F. J., de Keyzer, C. W., Diamond, S. E.,
461 Fortin, M. J., Frazee, L. J., Gorton, A. J., Hendry, A. P., Liu, Y., Losos, J. B., MacIvor, J.
462 S., Martin, R. A., McDonnell, M. J., Miles, L. S., Munshi-South, J., Ness, R. W., Newman,
463 A. E. M., Stothart, M. R., Theodorou, P., Thompson, K. A., Verrelli, B. C., Whitehead, A.,
464 Winchell, K. M., Johnson, M. T. J. (2019) A roadmap for urban evolutionary ecology.
465 *Evolutionary Applications* 12, 384-398. doi: 10.1111/eva.12734

466 Sacchi, R., Polo, M. S., Coladonato, A. J., Mangiacotti, M., Scali, S., Zuffi, M. A. L. (2018) The
467 exposition to urban habitat is not enough to cause developmental instability in the
468 common wall lizards (*Podarcis muralis*). *Ecological Indicators* 93, 856-863. doi:
469 10.1016/j.ecolind.2018.05.035

470 Schmitt, C. J., Cook, J. A., Zamudio, K. R., Edwards, S. V. (2018) Museum specimens of
471 terrestrial vertebrates are sensitive indicators of environmental change in the
472 Anthropocene. *Philos Trans R Soc Lond B Biol Sci* 374, 20170387. doi:
473 10.1098/rstb.2017.0387

474 Schneider, C. A., Rasband, W. S., Eliceiri, K. W. (2012) NIH Image to ImageJ: 25 years of
475 image analysis. *Nature Methods* 9, 671-675. doi: 10.1038/nmeth.2089

476 Snell-Rood, E. C., Wick, N. (2013) Anthropogenic environments exert variable selection on
477 cranial capacity in mammals. *Proceedings of the Royal Society of London B: Biological*
478 *Sciences* 280, 20131384. doi: 10.1098/rspb.2013.1384

479 Szulkin, M., Munshi-South, J., Charmantier, A. (2020) *Urban evolutionary biology*. Oxford
480 University Press, Oxford, UK

481 Teixeira, C. P., Hirsch, A., Perini, H., Young, R. J. (2006) Marsupials from space: Fluctuating
482 asymmetry, geographical information systems and animal conservation. *Proceedings of*
483 *the Royal Society B: Biological Sciences* 273, 1007-1012. doi: 10.1098/rspb.2005.3386

484 Tschardtke, T., Klein, A. M., Kruess, A., Steffan-Dewenter, I., Thies, C. (2005) Landscape
485 perspectives on agricultural intensification and biodiversity – ecosystem service
486 management. *Ecology Letters* 8, 857-874. doi: 10.1111/j.1461-0248.2005.00782.x

487 United Nations Population Division (2018). *World Urbanization Prospects: The 2018 Revision*.
488 United Nations.

489 Van Valen, L. (1962) A study of fluctuating asymmetry. *Evolution* 16, 125-142. doi:
490 10.1111/j.1558-5646.1962.tb03206.x

491 Weller, B., Ganzhorn, J. U. (2004) Carabid beetle community composition, body size, and
492 fluctuating asymmetry along an urban-rural gradient. *Basic and Applied Ecology* 5, 193-
493 201. doi: 10.1078/1439-1791-00220

494 Winchell, K. M., Briggs, D., Revell, L. J. (2019) The perils of city life: Patterns of injury and
495 fluctuating asymmetry in urban lizards. *Biological Journal of the Linnean Society* 126, 276-
496 288. doi: 10.1093/biolinnean/bly205

497 Zakharov, V. M., Yablokov, A. V. (1990) Skull asymmetry in the Baltic grey seal: Effects of
498 environmental pollution. *Ambio* 19, 266-269.

499

500 **FIGURE LEGENDS**

501

502 **Figure 1.** Points used to measure skull lengths. Dorsal (A) and ventral (B) pictures are from the
503 same representative *Mus musculus* specimen (Museum ID 21744). A) 1: anterior
504 intersection of the nasal bones; 2: posterior end of the skull; 3: bregma (intersection of
505 frontal bones and parietal bones at midline); 4: external point in the intersection of bones
506 in the zygomatic arch; 5: rostral end of the zygomatic arch; 6: caudal point in the
507 intersection of nasal bones; 7: posteriormost point of the orbital space. B) 8:
508 posteriormost point of the suture between palatines and the anterior border of the
509 mesopterygoid fossa; 9: posteriormost point of the incisive foramen; 10: anteriormost
510 point of the incisive foramen; 11: posteriormost point of the orbital space; 12:
511 anteriormost point of the foramen magnum.

512

513 **Figure 2.** Fluctuating asymmetry (FA) index values for the morphological measurement
514 MIDVEN in urban (N = 38) and rural (N = 23) specimens of *Mus musculus* in and around
515 the city of Vienna, respectively, between 1980 and 2013. For each boxplot, the bar
516 within each box represents the median, each box represents the first and third quartiles
517 (or 25th and 75th percentiles), the two whiskers represent the maximum values that are
518 within 1.5 * IQR of the box (where IQR or inter-quartile range is the distance between the
519 first and third quartiles), and the point beyond the whisker represents an outlier. The
520 significant difference between rural and urban specimens was obtained using a linear
521 model (** denotes $p = 0.0015$).

522

523 **Figure 3.** Fluctuating asymmetry (FA) index values for the morphological measurement
524 POSDOR over time and for 14 different rodent species. All specimens were captured in

525 rural areas in Austria. The line and shaded area represent the fit from a linear model and
526 a 95% confidence interval, respectively. FA values are log-transformed.