

# Morphological Stability of Rural Populations Confirms Their Use As Controls in Urban Ecology Studies

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## Research Article

**Keywords:** Anthropocene, fluctuating asymmetry, rodents, skulls, urban ecology, urban vs rural comparisons

**Posted Date:** September 13th, 2021

**DOI:** <https://doi.org/10.21203/rs.3.rs-795506/v1>

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# Abstract

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

## Introduction

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

The implicit assumption with rural vs urban comparisons is that the rural populations offer a control that represents a lack of the anthropogenic stressors affecting the urban populations. However, the reach of human influence in non-urban habitats is pervasive, particularly in the rural areas adjacent to cities, including increases in intensive agriculture; use of fertilizers and pesticides; habitat deforestation, fragmentation, and degradation; and road networks (Coda et al. 2016; Geiger et al. 2010; Marchand et al. 2003; Tschamntke et al. 2005). Anthropogenic disturbance in rural areas alters the distribution and abundance of native species and communities, which exacerbates the impacts that habitat disturbances

have on biodiversity (Ewers & Didham 2006; Geiger et al. 2010). However, it is unclear whether such anthropogenic disturbances significantly impact the phenotype of rural animals (Coda et al. 2016).

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

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

Morphological features in museum specimens can be used to assess the effect of environmental stressors in rural populations over time. In this context, measuring developmental instability is particularly fitting (Leamy & Klingenberg 2005). Developmental instability is the inability of an organism to produce a perfectly symmetrical phenotype because of environmental stressors affecting its normal development (Dongen 2006; Leamy & Klingenberg 2005). A frequently-used measure of developmental instability is fluctuating asymmetry (FA), defined as small, random deviations from perfect symmetry in a population (Leamy & Klingenberg 2005; Palmer & Strobeck 1986). FA is calculated by subtracting the value of a morphological feature on the right side of the body from the value on the left side of the body (Palmer 1994). These FA values will be negative in some individuals and positive in others, but they should not differ from zero at the population level (Dongen 2006; Van Valen 1962). Higher FA values indicate a higher incidence of environmental stressors affecting the normal development of organisms (Beasley et al. 2013). Consequently, FA of morphological traits has been widely used as an indicator of environmental and/or genetic stress driven by many different factors, e.g. inbreeding, extreme temperatures, food deprivation, parasitism, habitat fragmentation, or pollution (Beasley et al. 2013; De Coster et al. 2013; Lazić et al. 2013; Nunes et al. 2001). Importantly, FA has been used to study both the effect of urbanisation (Lazić et al. 2013; Winchell et al. 2019) and the effects of anthropogenic disturbance in rural areas (Coda et al. 2016; Marchand et al. 2003).

We used rodent skulls to conduct two separate studies. First, as a proof-of-concept we compared house mice from urban and rural regions in and around the city of Vienna since 1980. We predicted higher FA in urban mice than in rural mice. This study was intended to provide a baseline for the effect of urbanisation, to better interpret any changes over time in rural populations. Second, we determined if FA

in 14 rodent species has increased in rural areas over many decades. An increase in FA over time would indicate that rural populations have been noticeably affected by anthropogenic activity and that any rural vs urban comparisons may underestimate the effect of urbanisation. In contrast, a lack of changes in FA over time would support the common use of rural populations as a control in rural vs urban comparisons in urban ecology studies.

## Methods

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

For the urban vs rural study, we selected 61 adult specimens of *Mus musculus* that were collected in the city of Vienna or in rural sites nearby Vienna since 1980 (N = 23 from rural sites, and N = 38 from urban sites; see Supplementary dataset 1 for sex and location information). For the second study, we selected adult specimens (juvenile or adult status was based on information available in the museum records) that were collected in rural areas in Austria. If many specimens of a species were available for a short timeframe, we chose a subsample with a similar representation of males and females, in order to obtain a representative coverage of collection years for that species. Across all species, specimens were collected from 1824 to 2014 (maximum range was 1824 to 2006 in *Arvicola terrestris*, and minimum range was 1927 to 1998 in *Muscardinus avellanarius*). In total, we selected 495 specimens from 14 species: 39 *Apodemus flavicollis*; 46 *Apodemus sylvaticus*; 38 *Arvicola terrestris*; 35 *Cricetus cricetus*; 29 *Glis glis*; 24 *Micromys minutus*; 48 *Microtus arvalis*; 33 *Microtus subterraneus*; 16 *Muscardinus avellanarius*; 52 *Mus musculus*; 36 *Myodes glareolus*; 35 *Rattus norvegicus*; 27 *Sciurus vulgaris*; and 37 *Spermophilus citellus* (see Supplementary dataset 2 for sex, year of capture, and location information). Investigating several species is important, because changes in FA may be species-specific and driven by different ecological requirements (Coda et al. 2016).

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

Photographs were analysed using ImageJ 1.51s (Schneider et al. 2012). We used the ruler photographed next to the skull to set up the scale. We measured the total length of the skull, from the anterior intersection of the nasal bones to the posterior end of the skull (from point 1 to 2 in Fig. 1A, respectively). We also measured four dorsal and four ventral lengths in both the right and left sides of the skull. The four dorsal lengths were: BZA (Bregma-Zygomatic Arch), from bregma (intersection of frontal bones and parietal bones at midline; point 3 in Fig. 1A) to the external point in the intersection of bones in the zygomatic arch (point 4 in Fig. 1A); NASAL, from the anterior intersection of the nasal bones to the most

anterior point of the zygomatic arch (from point 1 to 5 in Fig. 1A, respectively); NAZ (NASal-ZygomatiC), from the caudal point in the intersection of nasal bones (point 6 in Fig. 1A) to the rostral end of the zygomatic arch (point 5 in Fig. 1A); and POSDOR (POSterior and DORSal), from the posterior end of the skull to the posterior end of the orbital space (from point 2 to 7 in Fig. 1A, respectively). The four ventral lengths were: CENVEN (CENTral and VENtral), from the posteriormost point of the suture between palatines and the anterior border of the mesopterygoid fossa (point 8 in Fig. 1B) to the posteriormost point of the incisive foramen (point 9 in Fig. 1B); FORAMEN, from the posteriormost point of the incisive foramen to the anteriormost point of the incisive foramen (from point 9 to 10 in Fig. 1B, respectively); MIDVEN (MIDsection and VENtral), from the posteriormost point of the suture between palatines and the anterior border of the mesopterygoid fossa (point 8 in Fig. 1B) to the posteriormost point of the orbital space (point 11 in Fig. 1B); and POSVEN (POSterior and VENtral), from the anteriormost point of the foramen magnum (point 12 in Fig. 1B) to the posteriormost point of the orbital space (point 11 in Fig. 1B).

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

## Statistical Analysis

We used R version 3.5.3 (R-Core-Team 2019) for all analyses. To check for the existence of FA and directional asymmetry (DA) for each one of the variables, we used mixed models (Palmer 1994). DA is an asymmetry that consistently favours one side of the body over the other, e.g. the location of the mammalian heart (Van Valen 1962). We log-transformed all measurements and fit a mixed model for each variable and for each species, using the function `lmer` (package `lmerTest`). In these models, we included side (right or left side of the skull) as a fixed effect, the interaction between side and individual, and individual as a random effect. A statistically significant difference between the right and left side across individuals would indicate DA. A statistically significant interaction between side and individual would indicate that the measurement for the right side was larger than the left side for some individuals but the opposite was true for other individuals, i.e. an indication of FA for that variable. For the first study (comparing rural vs urban specimens), we detected significant DA for the variables BZA, NASAL, and POSDOR, so we did not include these variables in the main analyses.

For the second study (rural specimens from several species over time), we detected significant DA for the variables NAZ, BZA, MIDVEN, and FORAMEN for several species, so we did not include these variables in the main analyses. Therefore, we considered the following morphological variables in the main analyses: NASAL, POSDOR, POSVEN, and CENVEN.

To assess fluctuating asymmetry in the main analyses, we first calculated a fluctuating asymmetry (FA) index for each individual and for each variable. In each case, we considered 4 values, i.e. the two replicated measures for each side. First, we calculated the mean for each side using the two replicated measures. Second, we subtracted the left value from the right value and used the absolute value from this subtraction as the FA index. Third, we log-transformed the FA index. We also calculated the mean skull length using the two replicated values for each individual (datasets with FA values are provided as supplementary material). For each variable we implemented a linear model using the *lm* function, the FA values being the response, and including type of habitat (urban or rural), sex, and year of capture as factors. We also included skull length as an offset. In the analyses assessing FA values of several species over time in rural locations, we also included species and the interaction between species and year of capture as factors in the models.

## Results

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

In the study considering rural specimens over a large period of time, we found species-specific differences in FA for the four considered morphological measurements (CENVEN:  $p < 0.0001$ ; NASAL:  $p = 0.028$ ; POSDOR:  $p = 0.028$ ; and POSVEN:  $p = 0.004$ ). However, pairwise comparisons between species (using the Tukey method for the adjustment of p values with the function *emmeans* from the package *emmeans*) were only statistically significant for CENVEN, with FA values from *Arvicola terrestris* being significantly higher than those from *Apodemus sylvaticus*, *Apodemus flavicollis*, *Mus musculus*, *Micromys minutus*, *Microtus subterraneus*, *Microtus arvalis*, *Myodes glareolus* and *Spermophilus citellus* ( $p < 0.05$  for all analyses), and FA values from *Cricetus cricetus* being significantly higher than those from *Apodemus flavicollis*, *Micromys minutus*, *Microtus subterraneus* and *Myodes glareolus* ( $p < 0.05$  for all analyses). Sex and the interaction between species and year of capture did not affect FA values for any of the morphological measurements ( $p > 0.05$  for all analyses). Importantly, FA values did not differ by year of capture in the case of 3 morphological measurements (CENVEN, NASAL, and POSVEN:  $p > 0.05$  for all analyses), and FA values for POSDOR significantly decreased over time ( $F_{1,423} = 10.59$ ,  $p = 0.0012$ ; Fig. 3).

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

## Discussion

We found a higher incidence of FA in the midsection of the skull in urban house mice compared to rural house mice. However, we did not detect an increase in FA in rural populations of several rodent species over the last two centuries, which supports the common practice in urban ecology studies of using rural populations as a control in rural vs urban comparisons when assessing the effects of urbanisation.

Several studies have found increased levels of asymmetry in response to different types of anthropogenic disturbance, such as high levels of urbanisation, in many organisms, including plants, invertebrates and vertebrates (Banaszak-Cibicka et al. 2018; Cuevas-Reyes et al. 2013; Eeva et al. 2000; Elek et al. 2014; Lazić et al. 2013; Teixeira et al. 2006; Weller & Ganzhorn 2004). Our finding of higher asymmetry in the skulls of urban house mice in Vienna compared to rural specimens adds to this literature, and offers further support to the use of FA as an useful indicator of stress experienced by organisms in urban environments. It must be noted however that there are some conflicting results in the use of FA to assess the effect of urban stressors. For example, in carabid beetles, FA increased with urbanisation in species considered to be negatively affected by urbanisation, whereas such a relationship was not found in species that are more tolerant to urbanisation (Weller & Ganzhorn 2004); however, in another study FA did not change along rural-urban gradients (Elek et al. 2014). Similarly, different studies in lizards have reported an increase in FA in response to urbanisation (Lazić et al. 2013), no changes in FA in response to urbanisation (Sacchi et al. 2018), and even a decrease in FA in response to urbanisation, the authors interpreting this last result as natural selection being stronger in urban populations and asymmetrical individuals being less likely to survive to adulthood (Winchell et al. 2019).

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

Traits that have a direct impact on survival or fitness, e.g. extremities and traits involved in foraging, are under strong selection and may be developmentally more stable (De Coster et al. 2013) and thus not reflect the effects of environmental impacts particularly well (Clarke 1995; Dongen 2006; Winchell et al. 2019). In contrast, traits for which some level of asymmetry may not necessarily result in a decrease in fitness may be better indicators of the environmental stress experienced by individuals during development (Clarke 1995). Although it is unclear whether small skull asymmetries may directly decrease fitness, there are nonetheless several studies reporting that poor environmental conditions increase skull asymmetry (Maestri et al. 2015; Marchand et al. 2003; Oleksyk et al. 2004; Zakharov & Yablokov 1990).

In our study, the lack of an increase in FA over the last decades in rural areas was consistent across different species with different ecological requirements and thus possibly affected differently by any given anthropogenic activity. This validates our main conclusion that rural populations have been unaffected by anthropogenic activities in an increasing manner over the last centuries. For example, rodent species closely associated to bodies of water (e.g. *Arvicola terrestris*, the European water vole) may be more affected by fertilisers and other contaminants reaching and accumulating in those water bodies. We selected a broad array of species with different ecological requirements, including commensal species (*Mus musculus*, house mouse; *Rattus norvegicus*, brown rat; and *Apodemus sylvaticus*, wood mouse), species associated to water bodies (e.g. *Arvicola terrestris* and *Rattus norvegicus*), species associated with grasslands (e.g. *Micromys minutus*, Eurasian harvest mouse; and *Microtus arvalis*, common vole), species mainly associated to woodlands (e.g. *Myodes glareolus*; *Apodemus flavicollis*, yellow-necked mouse; *Glis glis*, edible dormouse; *Muscardinus avellanarius*, hazel dormouse; and *Sciurus vulgaris*, Eurasian red squirrel), species restricted to low-land areas where they are likely to be exposed to diverse anthropogenic activities (e.g. *Cricetus cricetus*, the common or European hamster), or species restricted to areas with soft turf where they can dig burrow systems (*Spermophilus citellus*, European ground squirrel). Despite these ecological differences, we did not find any increase in FA over time in any individual species. We found, however, that two species (*Arvicola terrestris* and *Cricetus cricetus*) had significantly higher FA than many other species, regardless of year of capture. These two species are closely exposed to pollution (e.g. pesticides) given their habitat preferences, but so are other species included in our analyses, so it is unclear why FA values were higher in these particular species. Similarly, the species differences that we observed cannot be explained through a distinction between specialist and generalist species (Coda et al. 2016; Teixeira et al. 2006).

Our study emphasises the importance of museum collections to address questions that may not be easily tackled otherwise. By using museum specimens collected over the last centuries we can investigate how populations respond over time to changing environmental conditions, e.g. the impact of anthropogenic disturbance (Pergams & Lacy 2008; Pergams & Lawler 2009; Snell-Rood & Wick 2013).

In conclusion, our study offers some general validation to the use of rural populations as an appropriate control when investigating the effect of urbanisation on urban populations, with the caveats that this may not be the case for all species, and that there may be abiotic and ecological factors uniquely affecting rural habitats. That is, rural habitats cannot be defined by the absence of human disturbance. Ultimately, any comparison of habitats should quantify the factors affecting each habitat and the impact that those factors may have on the populations under study.

## Declarations

### ACKNOWLEDGEMENTS

We thank Frank Zachos and Alexander Bibl for facilitating access to specimens and databases in the Mammal Collection at the Natural History Museum Vienna, and for their assistance locating specimens.

We also thank Kathryn Simmons for assistance photographing specimens at the Natural History Museum Vienna.

**Funding.** This work was supported by a Synthesis grant from the European Commission (grant number AT-TAF-6333).

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

**Availability of data and material.** Datasets are provided as supplementary material.

**Code availability.** R script available from the corresponding author.

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

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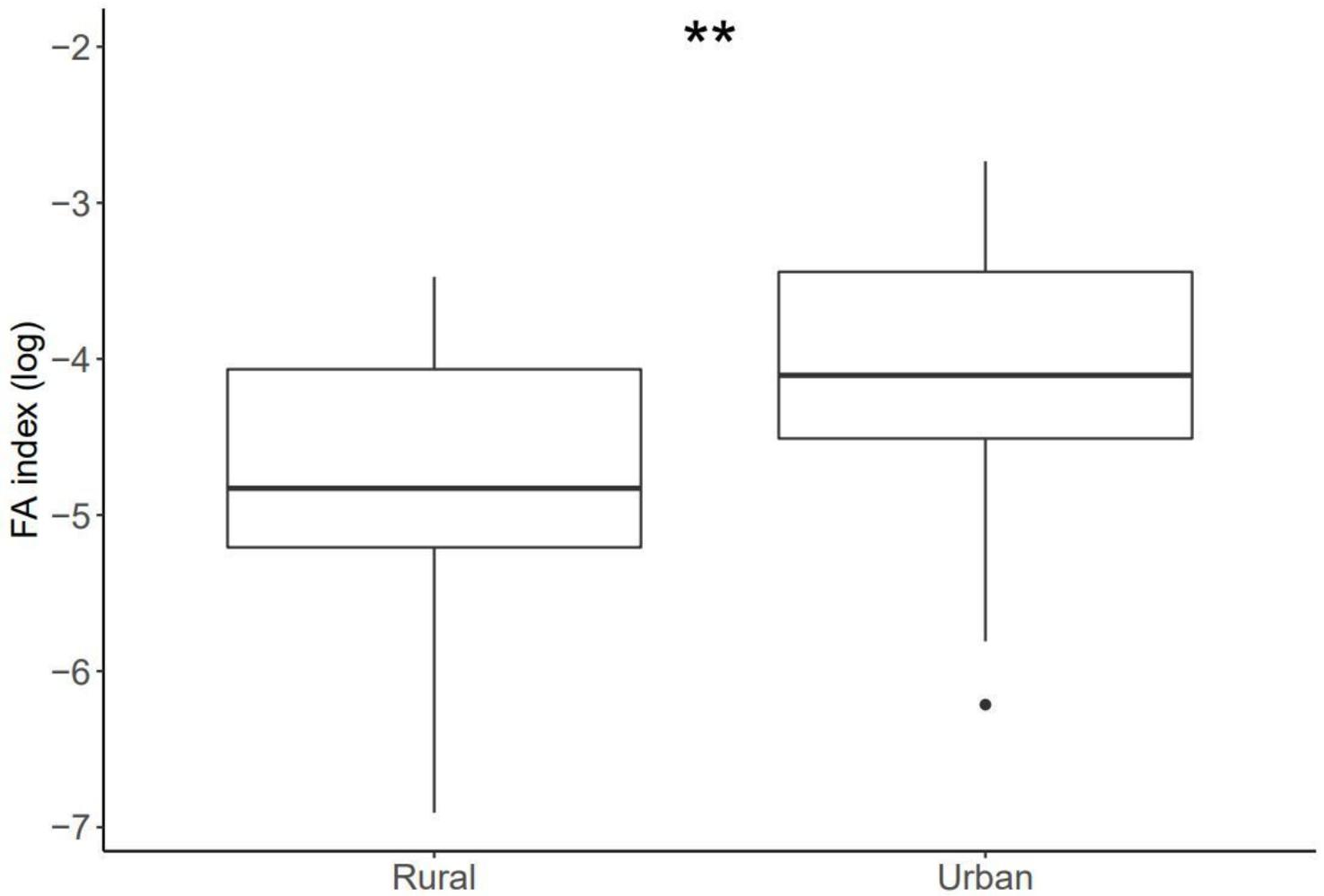
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## Figures



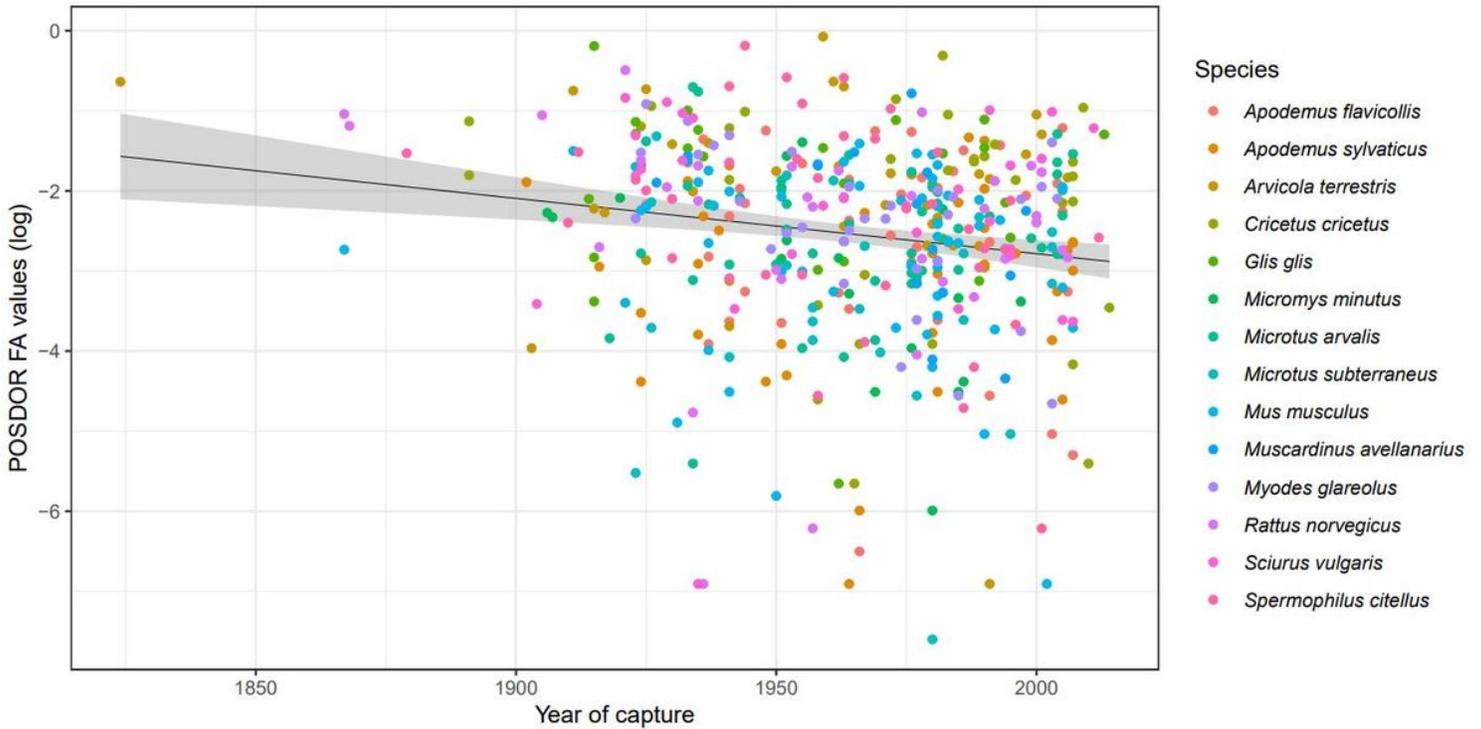
**Figure 1**

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



**Figure 2**

Fluctuating asymmetry (FA) index values in urban (N = 38) and rural (N = 23) specimens of *Mus musculus* in and around the city of Vienna, respectively. For each boxplot, the bar within each box represents the median, each box represents the first and third quartiles (or 25th and 75th percentiles), the two whiskers represent the maximum values that are within 1.5 \* IQR of the box (where IQR or interquartile range is the distance between the first and third quartiles), and the point beyond the whisker represents an outlier. The significant difference between rural and urban specimens was obtained using a linear model (\*\* denotes  $p < 0.005$ ).



**Figure 3**

Fluctuating asymmetry (FA) values for the morphological measurement POSDOR over time and for 14 different rodent species. All specimens were captured in rural areas in Austria. The line and shaded area represent the fit from a linear model and a 95% confidence interval, respectively. FA values are log-transformed.

## Supplementary Files

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