Urbanisation drives genetic differentiation in physiology and structures the evolution of pace-of-life syndromes in the water flea *Daphnia magna*

Running title: Urban stress physiology and pace-of-life

Kristien I. Brans[1]*, Robby Stoks[2] & Luc De Meester[1]

[1]Laboratory of Aquatic Ecology, Evolution and Conservation, KU Leuven, Ch. Deberiotstraat 32, B-3000 Leuven, Belgium

[2]Laboratory of Evolutionary Stress Ecology and Ecotoxicology, KU Leuven, Ch. Deberiotstraat 32, B-3000 Leuven, Belgium

*Corresponding author: kristien.brans@kuleuven.be, +3216373846

Key words: urbanisation, protein content, fat content, oxidative stress responses, pace-of-life syndrome, urban evolution

A**bstract (200)**

Natural and human-induced stressors elicit changes in energy metabolism and stress physiology in populations of a wide array of species. Cities are stressful environments that may lead to differential selection on stress-coping mechanisms. Given that city ponds are exposed to the urban heat island effect and receive polluted run-off, organisms inhabiting these ecosystems might show genetic differentiation for physiological traits enabling them to better cope with higher overall stress levels. A common garden study with 62 *Daphnia magna* genotypes from replicated urban and rural populations revealed that urban *Daphnia* have significantly higher concentrations of total body fat, proteins, and sugars. Baseline activity levels of the antioxidant defense enzymes superoxide dismutase (SOD) and glutathione-S-transferase (GST) were higher in rural compared to city populations, yet urban animals were equally well protected against lipid peroxidation. Our results add to the recent evidence of urbanisation-driven changes in stress physiology and energy metabolisms in terrestrial organisms. Combining our results with data on urban life history evolution in *Daphnia* revealed that urban genotypes additionally show a structured pace-of-life syndrome involving both life history and physiological traits, whereas this is absent in rural populations.

INTRODUCTION

Cities currently stage one of the most remarkable shifts in species occurrence and loss, community structure, biotic homogenization, and rapid adaptive evolution [1-6]. Multiple stressors, such as pollution, drought, heat, and changed biotic interactions act in concert in cities [7], generating an environment that is thought to select for those species and phenotypes able to cope with higher overall stress levels [e.g. 8-12].

Maintaining homeostasis heavily depends on the balance between oxidative damage and antioxidant repair mechanisms [13, 14]. Oxidative damage to lipids, proteins, and DNA can be caused by pro-oxidative by-products of respiration (reactive oxygen and nitrogen species, ROS and RNS; [14]). This leads to oxidative stress, a state in which cellular antioxidant and repair mechanisms fail to quench excess levels of ROS/RNS [13, 14]. Natural stressors, such as predation, overcrowding (competition), food stress (low food quantity or quality), oxygen depletion, and temperature extremes are known to cause enzymatic and oxidative trait changes linked to stress resistance [14, 15-18]. In cities, these environmental stress factors are disproportionally altered due to human activity [7]. Additionally, non-natural perturbations linked to pollution (e.g. metals, pesticides, noise, and light pollution) have been shown to change redox states in terrestrial and aquatic animals due their high oxidative reactivity with biomolecules [19-22]. Therefore, it might be expected that city populations feature adaptive changes in antioxidant defense mechanisms [10, 23, 24].

Studies on stress physiology of urban species and populations have only started to emerge, but evidence is rapidly growing that urbanisation has a significant impact on the regulation of oxidative stress machinery (e.g. antioxidant enzymes, immune function, stress-

related behavioral changes, and energy metabolism [8, 10, 12, 23, 25-28]. For example, field-caught urban house sparrows have higher activity of antioxidant enzymes (SOD and GST), yet lower total antioxidant capacity (TAC) and higher levels of oxidative damage in their blood compared to rural birds [28]. Given the taxon-specificity of life histories, dispersal mode, and preferred environmental conditions, as well as the interplay with non-physiological stress-coping mechanisms (e.g. avoidance behavior), a straightforward general link between levels of urbanisation and the up- or downregulation of antioxidant activities, or oxidative damage, has proven difficult to confirm [11, 12, 24, 29, 30]. Additionally, energy reserve compounds such as fat, sugars, and proteins can be an important component of stress responses, as they provide cellular energy that can be allocated to growth and reproduction, active dispersal, or an increase in physiological stress responses, and thereby provide the fuel to cope with stressful conditions and enable population survival [11, 32]. For instance, field caught juvenile house sparrows contain higher levels of body fat in urban than in rural areas [11].

Small aquatic ecosystems such as ponds located in the city are warmer [3, 33] and often receive organic and chemical pollutants such as road salts, nutrients, metals, and pesticides via surface run-off or atmospheric depositions [34, 35]. In addition, they are often characterized by higher disturbance levels (e.g. water level control, leaf litter cleaning [34]). As populations of plants and animals inhabiting urban ponds might be exposed to greater environmental stress, we hypothesize that urban environments select for different coping mechanisms at the physiological level, causing genetic differentiation between rural and urban populations in traits such as energy storage molecules (e.g. fat, proteins, and sugars), and oxidative stress enzymes (CAT, SOD, GST). Although some studies observed clear phenotypic upregulation of stress physiological

88 parameters (e.g. GST: [36]; Hsp70: [31]), evidence for an evolutionary shift in organismal stress
89 physiology in cities is so far limited (but see [birdsref partecke costantini].

90 To quantify the degree of urbanisation-driven genetic differentiation in stress physiology,
91 we set out a common garden experiment with urban and rural genotypes of *Daphnia magna*,
92 originating from replicated urban and rural populations (Flanders, Northern Belgium). In earlier
93 work, *Daphnia* from city populations were found to mature faster and at a smaller size, produce
94 more offspring and release their juveniles sooner, resulting in a higher intrinsic population
95 growth rate and a shift along the pace-of –life axis towards a faster life style compared to rural
96 populations (Brans et al., in revision; Fig. S3, Supplementary Information A). In a common
97 garden experiment, we here measured seven physiological response variables that are either
98 linked to energy metabolism (total fat, sugar, and protein content) or oxidative stress responses,
99 quantifying both antioxidative defense (activity of SOD, CAT, and GST) and damage
100 (concentration of malondialdehyde, a by-product of lipid peroxidation). We reared animals at
101 two different temperatures to mimic the urban heat island reported to occur in these ecosystems
102 [3, 33], enabling us to quantify phenotypic plasticity across two temperatures and assess habitat-
103 specific (urban versus rural) phenotypic plasticity in the measured stress physiological responses.
104 We tested the hypothesis that there is genetic differentiation between urban and rural *Daphnia*
105 populations for these physiological responses. More specifically, we expected that urban *Daphnia*
106 might be characterized by higher amounts of storage molecules that can be invested in faster
107 growth and development, or in detoxification processes. In analogy with other studies showing
108 that populations from stressful habitats are often genetically characterized by lower basal
109 expression of stress proteins (REF), we also expected that urban populations might exhibit lower
110 basal expression levels of antioxidant defense enzymes (SOD, CAT, GST), while suffering a

similar or even reduced level of oxidative damage. We additionally tested for covariation patterns to explore to what extent the studied life history and physiological traits are shaped by an overall pace-of-life syndrome [37]. The Pace-of-life Syndrome (POLS) hypothesis [37] predicts a concerted change in both life history and physiological traits, as the physiological traits determine how much energy can be invested in life history features [66]. The presence and structure of covariation patterns has been suggested to be environment-dependent and potentially under selection [e.g. 67, 68]. Using multi-group structural equation modelling (SEM) on urban and rural *Daphnia*, we tested whether pace-of-life-syndrome covariation patterns are dependent on urbanisation background (evolution) or rearing temperature (plasticity) [38-40].More specifically, we hypothesize that especially in urban populations, , a close link between life history and physiology might have evolved, where physiological processes (e.g. energy budget) are aligned to support their faster life style.

MATERIAL AND METHODS

Urbanisation gradient, model organism, and experimental design

We sampled 13 *Daphnia magna* populations along multiple well-characterized urbanisation gradients in Flanders (Northern Belgium; see Fig. S1, Supplementary Information A), with the percentage built-up area (BA; available via the Large-scale Reference Database at Flanders Geographical Information Agency) in the regional surroundings of the pond (3200 m radius) as a proxy for the degree of urbanisation. Given that this measure does not include roads and parking lots, an area with a BA of 10% and more is considered highly urbanized. We classified all ponds according to their %BA into rural (< 5%, n =5) and urban (> 10%; n = 7). Rural ponds were additionally characterized by a minimum of 20% of Biologically Valuable Area in a 3200 m radius to ensure that they would be situated in (semi)natural areas and not in areas characterized by intensive agriculture (cf. pollution with pesticides and agricultural run-off). We performed all experiments with clones of these 13 populations, but due to accidental thawing we lost the set of samples of highly urbanized population *Brxl*, which prevented assessing physiological traits for this population.

Daphnia magna has a cyclic parthenogenetic life cycle; under favourable conditions all-female clonal lineages can be established and cultured over several generations. Six females from each population were isolated and reared under standardized laboratory conditions (dechlorinated tap water, 80% water refreshment every two days, 20 °C, 14:10h L:D photoperiod, daily fed 10^5 cells.ml^{-1} of the green algae *Acutodesmus obliquus*; for more detailed information on culturing conditions we refer to [3, 41] and Supplementary Information B). After one generation of culturing under standardized conditions, 2nd brood juveniles from each line were split up and divided over six replicated cultures (i.e. 36 cultures per population, total number of experimental

146 units: 432), later to be assigned to the appropriate temperature treatment. We screened clonal
147 identity of all lines using microsatellite markers (27 markers, detailed information in [3]), which
148 was added as an additional level of within-population variation in the statistical analysis (i.e.
149 'clone', Table S1, Supplementary Information A). All population x line x temperature replicates
150 were cultured for an additional two generations under the same standardized laboratory
151 conditions to purge (grand)maternal effects, after which we transferred a cohort of 12 juveniles (<
152 24h old) of each line to the appropriate temperature treatment (20 °C/ 24 °C).

153 We carried out a cohort life table experiment with all lineages at two temperatures, 20 and
154 24°C, reflecting the average summer temperature in rural and urban populations, respectively
155 (Brans et al. 2018, Supplementary Information xxxx).

156 Cohorts were followed up to assess thermal tolerance and measure life history traits
157 (reported on in [41] and Brans et al., in revision). After release of the 2nd clutch, six animals from
158 each experimental cohort were pooled and shock frozen in liquid nitrogen until sample
159 processing. Body length (from center of eye to base of tail spine) of three animals of each pooled
160 sample was measured (Olympus X stereo microscope), and average length was translated into dry
161 mass using a published length-weight regression [42] so as to standardize measured amounts of
162 proteins, fat, carbohydrates and MDA by dry weight. Protocol and sample analysis for the
163 quantification of response variables are detailed in Supplementary Information, section B.

164 The pooled sample of animals per replicate of a clone-by-treatment combination was
165 homogenized with a pestle in PBS buffer (Phosphate-Buffered Saline, 50 mM, pH: 7.4, 50
166 µl/animal) and centrifuged for 8 min at 13000 rpm and 4 °C. All response variables were
167 measured on the resulting supernatant using a Tecan plate reader. Detailed sample analysis

protocols are given in Supplementary Information (section B). In brief, t otal protein content was quantified following the Bradford protocol ([43]; in quadruplicate), total fat content following Bligh & Dyer [44], and total sugar content (glucose + glycogen) following Stoks et al. [45]. The activity of superoxide dismutase (SOD), catalase (CAT), two key antioxidant enzymes in invertebrates [13], and gluthation-S-transferase (GST), a secondary antioxidative enzyme that protects against oxidizing and toxic substances by detoxifying ROS-damaged cellular components, was measured as an estimate of antioxidant defense. SOD and CAT activity was quantified using the protocol of De Block & Stoks [46], , while GST activity was quantified following McLoughlin et al. [47]. Oxidative damage was quantified as malondialdehyde (MDA) concentration [48], a byproduct of lipid peroxidation, following the protocol of xxxxx. Total fat content was used as a covariate in the statistical analysis.

Statistical analysis

To test for differentiation in physiological traits between urban and rural *Daphnia* (categorical), and between animals reared at 20 °C and 24 °C (categorical), we first performed a MANOVA, followed by separate linear mixed-effect models (using the R software, [49]) on all response variables scaled to a mean of 0 and standard deviation of 1 (fixed effects: urbanisation, temperature, and their interaction). Population and clone within populations were implemented as random variables. Model specifications, random structure, computed test statistics, and the validation of model assumptions are detailed in Supplementary Information (section B). Significance of 'clone' reflects evolutionary potential within populations.

We tested for the impact of urbanisation and/or temperature on covariation between life history and physiological traits along the pace-of-life axis (cf. Pace-of-life Syndrome Hypothesis [37]) by combining the stress physiological traits with data on eight life history traits measured

191 on the same cohorts of individuals in a life table experiment (Brans et al., in revision). *Daphnia*
192 genotypes from urban populations show an evolutionary shift in life style towards the fast end of
193 the pace-of-life continuum compared to *Daphnia* genotypes from rural populations (see Fig. S3,
194 Supplementary Information A). Using structural equation modelling [50], we explored to what
195 extent the shift towards a faster life style in urban populations covaries with trait shifts in stress
196 physiology according to an overarching pace-of-life syndrome [37], and whether urbanisation
197 (evolution) and/or temperature treatment (plasticity) shape these POLS trait covariation patterns
198 in a consistent manner [38, 51].

199 Using multi-group SEM [52], we first evaluated the consistency of trait covariation
200 patterns at both levels of biological organization separately (i.e. life history trait covariation
201 patterns and physiology trait covariation patterns, independent of each other; paths a-i and j-p,
202 respectively, in Fig. S4, Supplementary Information A). Second, we evaluated consistency of trait
203 variation in an overarching pace-of-life syndrome model integrating life history and physiology
204 (paths q and r, Fig. S4). The rationale behind each SEM is that a 'latent' (i.e. non-measured)
205 variable can be incorporated in the model (Fig. S4, ovals) that structures the covariation patterns
206 observed in the measured variables (squared boxes). The suits of traits linked in a 'syndrome'
207 given by the latent variable are indicated via significant pathways [50]. Each pathway in the SEM
208 represents a linear model. For significant paths, a change in one variable (standardized units for
209 all variables) coincides with a change in all other traits of the syndrome (according to path
210 coefficients, which represent standardized model estimates). Multi-group SEM allows to compare
211 models with factor loadings that are constrained or freely varying across temperature treatment,
212 urbanisation level, or a combination of both (detailed information in Supplementary Information
213 B). This results in different model hypotheses (Model I to IV, Table S2, Supplementary

Information A). For example, if the best model fit is characterized by factor loadings being constrained across temperature treatments, but not urbanisation level (model II), this indicates that trait covariation patterns significantly differ between urban and rural populations but are the same for animals reared at 20 and 24 °C (and vice versa for model III). This would imply that urbanisation background, and thus evolution (or temperature and thus plasticity, for model III), significantly shaped trait covariation patterns. Models were ranked according to AIC scores, model weights (*W*), and evidence ratios (E.R.) (detailed in Supplementary Information, section B). In case models II, III, or IV were selected as best model (i.e. a non-fully restricted model), group differences of interest were compared by comparing factor loadings (significance, direction, and size) using the method of Zar ([53]; see also [38, 51], and Supplementary Information section B for calculations). All models were fitted with the package 'lavaan' [54] using the R software [49]. All variables were scaled and standardized prior to analysis.

RESULTS

*Genetic differentiation in energy reserves and stress physiology*MANOVA indicates an overall significant effect of urbanisation on the combined set of physiological response variables ($p < 0.001$, Table 1). General linear-mixed effect models on each variable separately reveal that urban animals contain significantly more fat ($p = 0.003$, Fig. 1a), sugars ($p < 0.001$, Fig. 1b), and protein ($p = 0.002$, Fig. 1c) per µg dry mass (Table 1), suggesting a higher amount of energy reserves in urban compared to rural populations. Animals reared at a higher temperature had less protein per µg dry mass ($p = 0.02$, Table 1) but did not show differences for fat and sugar content (Table 1).

Urban animals did not differ from rural ones in CAT activity ($p = 0.525$, Fig. 2a), but had a lower SOD ($p = 0.013$, Table 1, Fig. 2b) and GST ($p = 0.023$, Table 1, Fig. 2c) base line activity. MDA concentrations (lipid peroxidation) did not differ between urban and rural animals ($p = 0.835$, Fig. 2d). Elevated rearing temperature induced a significant increase in the activity of CAT ($p < 0.001$, Fig. 2a) and SOD ($p < 0.001$, Fig. 2b), but not in GST, and there were no differences in lipid peroxidation. We did not observe significant urbanisation x temperature interaction effects for any of the traits.

Structural equation modelling: exploring the Pace-of-life Syndrome Hypothesis

Life history trait covariation patterns

The path analysis on life history traits revealed a syndrome structure with underlying life history trait covariation differing according to model IV (Table S3, factor loadings all free; Fig. S5, Supplementary Information A), indicating differently structured life history trait covariation patterns across all four urbanisation x temperature treatment groups. This implies that both

urbanisation background (evolution) and temperature treatment (plasticity) significantly shape life history trait covariation.

Physiology trait covariation patterns

The best model explaining a structuring syndrome at the level of physiological traits was model I (Table S3, all factor loadings constrained to be equal: Fig. S6, Supplementary Information A), indicating a consistent trait covariation across all four urbanisation x temperature groups.

Overarching Pace-Of-Life Syndrome

In the overarching POLS models, model IV was the best supported, indicating that covariation patterns differ among all groups (Table S3). Both urbanisation background (evolution) and temperature treatment (plasticity) shape trait covariation patterns. The difference in trait covariation patterns between urban and rural animals reared at 20 °C reflects genetic differentiation for a POLS (Fig. 3a,b; significantly differing paths at the level of $p < 0.05$ are indicated with †; for the pattern at 24°C, see Fig. S7, Supplementary Information A). The major difference between urban and rural animals at 20 °C was that the overarching syndrome structure linking the physiological and life history syndromes was significant in the urban but not rural animals(Fig. 3a,b; paths q and r in Fig. S4, Supplementary Information A; both $p < 0.001$). This overarching POLS reflects that, within the set of urban animals, fast-paced (i.e. fast maturation, high fecundity, high population growth rate) animals have lower amounts of protein, fat, and sugars, but higher activity of oxidative stress enzymes and higher oxidative damage. This suggests that, within urban animals, shifts towards a faster life-style depletes energy resources and increases oxidative stress physiology. In the rural path model, no such link between physiology and life history, and thus no overarching POLS, is present (Fig. 3b). Within the nested

life history syndrome, fecundity (number of juveniles in both clutch 1 and 2) significantly contributed to the syndrome structure in rural but not in urban animals (both $p < 0.001$). When looking at the nested physiological syndrome, MDA significantly covaried with other physiological traits in urban but not in rural animals ($p < 0.001$), whereas GST covaried with other physiological variables in rural but not in urban animals ($p = 0.049$). Of the significant paths shared between both covariation models (i.e. paths significantly contributing to the syndrome structure in both urban and rural animals), all path coefficients had the same direction in both groups, but significantly differed in size between urban and rural genotype sets for development time (age at maturity, age at release of first and second clutch), fecundity (both clutches), somatic growth, total protein content, MDA, and SOD (Fig. 3a,b; indicated with †).

DISCUSSION

The results of our common garden experiment reveal pronounced genetic differences between urban and rural populations of the water flea *Daphnia magna* for a set of physiological traits linked to stress resistance and energy reserves. Compared to rural, urban *Daphnia* have higher concentrations of protein, fat, and sugars, reflecting a higher level of energy storage compounds available per unit dry mass. The activity of antioxidant enzymes superoxide dismutase (SOD) and glutathione-S-transferase (GST) was upregulated in rural animals, yet malondialdehyde (MDA) concentrations, a measure for lipid peroxidation and thus oxidative damage, did not differ between both genotype sets. These results suggest that urban *Daphnia* have evolved a more efficient stress resistance compared to rural animals. While raising the animals at 24 °C compared to 20 °C led to a reduction in total protein content and an increased activity of SOD and GST, we observed no urbanisation x temperature interactions. Evolutionary differentiation thus involved constitutive trait changes but no evolution of plasticity. Integrating our data on physiological traits with existing data on life history traits measured on the same set of genotypes reveal an overarching pace-of-life syndrome, structuring covariation patterns between life history and physiology in urban, but not in rural animals. Our results thus show that urbanisation not only induces evolution of mean trait values, but also shapes trait covariation patterns. More specifically, in urban animals life-history and physiology are integrated into a syndrome, covarying along the pace-of-life axis, while the two sets of traits were unlinked in rural animals. In the following paragraphs we discuss these results in more detail.

Genetic differentiation in energy reserves and stress physiology

Most urban ecological studies reporting on stress physiology focus on terrestrial vertebrates such as birds and mammals [12, 23, 25-27; but see 25 for amphibians]. As most

studies use field collected animals, they do not reveal whether or not the observed differences in physiological stress-coping mechanisms between urban and rural populations are driven by (adaptive) evolution(but see e.g. [55, 56]). Here we show that populations of the water flea *D. magna* inhabiting small ponds in urban areas have genetically diverged from those in rural areas. Urban *Daphnia* genotypes have higher concentrations of protein, fat, and sugars per unit of dry mass. Urban populations are therefore characterized by a higher available energy budget [57] than rural populations, which is important in determining their scope of growth under stress [57, 58]. Urban populations are also characterized by a reduced activity of antioxidant enzymes (SOD and CAT), while oxidative damage to lipids did not differ, indicative of more effective defense responses. These results are in line with earlier findings on the evolutionary and acclimatory responsiveness of *Daphnia* when exposed to human-associated toxicants such as heavy metals and pesticides [59-61] and natural toxicants such as cyanotoxins [62]. , The responses we observe most likely reflect adaptive evolution to cope with higher stress levels associated with anthropogenic disturbances in urban environments. While we have convincing data on differences in temperature in the studied urbanization gradients [33], the observed evolutionary divergence might also be related to mechanical disturbance, [34] or pollution. As we did not test for fitness differences between urban and rural populations, non-adaptive evolutionary processes cannot be ruled out as drivers of the observed genetic differentiation. Yet, given that we selected populations across multiple urbanisation gradients (multiple cities), genetic drift is unlikely the driving force, as genetic drift has no direction and is thus unlikely to result in such a highly repeatable pattern. The significant effects of clone that we observe furthermore indicate the presence of evolutionary potential for four out of seven traits measured.

The results of our study reinforce earlier observations on genetic differentiation in *D. magna* populations in response to urbanisation, which involved both heat tolerance [41] and life history traits (Brans et al., in revision). Combined, they suggest that urban populations of *D. magna* are genetically different from rural ones in multiple traits linked to warming, pace-of-life, and overall stress. Traits range from heat tolerance (i.e. survival at higher temperatures) to energy metabolism, oxidative stress responses, and a diverse set of life history features. The number of studies unequivocally showing evolution in response to urbanisationis rapidly growing (e.g. [3, 41, 63-65]), but none involved an as extended set of traits as reported for *Daphnia*. This broad set of responsive traits and the fact that it involves heat tolerance, with a direct link to temperature differences, provides further evidence for adaptive evolution as a driver of these differences.

The Pace-of-life syndrome Hypothesis: linking life history and physiology

Our earlier work on life history differentiation indicated that urban *D. magna* populations showed a clear-cut shift to a faster pace-of-life compared to rural populations. Due to energy allocation trade-offs, stress physiological responses are often closely intertwined with higher-level response traits such as behavioral or life history traits, potentially leading to a pace-of-life syndrome [36] structuring covariation patterns between life history and physiological traits. Strikingly, structural equation modeling revealed such an association between physiological and life history traits in urban but not in rural populations. In urban populations, animals with shorter maturation time and a faster release of the first clutch are characterized by an increase in somatic growth and intrinsic population growth rate, yet have a lower protein content, upregulated SOD and GST activity, and suffer higher lipid peroxidation. These covariation patterns suggest that, within the set of urban genotypes, investment in a faster life style depletes energy reserves and increases antioxidant defense activity (SOD), yet still comes at a cost of increased lipid peroxidation (MDA). We

353 hypothesize that the evolution of a faster pace-of-life (i.e. smaller size at maturity, faster
354 maturation and reproduction, increased fecundity) is a key feature of urban *D. magna* populations
355 (Brans et al., in revision), and that selection for an increased pace-of-life has been so strong that
356 physiological traits necessarily became aligned to facilitate the transition to a faster life style. The
357 fact that an underlying syndrome between life history and physiology is only present in the city
358 populations is in line with the hypothesis that such strong associations between suits of life
359 history, physiological or behavioral traits are more likely to occur in low-quality habitats or
360 stressful conditions (e.g. [38, 67-69]).

361 While within urban genotypes the POLS reflects that a faster life style comes at a cost of
362 reduced energy reserves and higher oxidative damage, we observe the opposite relation when
363 comparing urban to rural populations. Here, the faster life style of urban populations is associated
364 with higher investment in energy storage and with reduced investment in antioxidant defense.
365 The evolution of oxidative stress responses in urban populations is, similar to the observations
366 made in many other studies (reviewed in [11]), not consistent across all three enzymes tested
367 (Table 1; Fig. 2). Overall, however, there is a reduction in oxidative stress enzyme activity in
368 urban compared to rural populations, while there is no difference in oxidative damage, suggestive
369 of a more efficient defense mechanism. These results are at first sight counter-intuitive given that
370 urbanisation induces a faster pace-of-life, which is expected to result in a higher metabolic rate
371 and associated increase in oxidative stress [66, 72]. Oure results might, however, reflect that
372 urban populations are adapted to an overall more stressful environment through a reduced
373 metabolic rate and investment in defense proteins under baseline conditions, as has been
374 repeatedly observed in a variety of organisms (REFS). This might be achieved through the
375 evolution of more efficient defense mechanisms or through trade-offs with other traits, such a

antipredator defense or immune function (REF). Within the set of urban genotypes, however, variation in traits among genotypes does reflect a classic trade-off between faster life and reduced energy reserves and increased oxidative stress

Stress in the city leading to adaptive evolution?

We documented genetic differentiation of *D. magna* populations along urbanisation gradients, which represent multifarious selection gradients, City ponds are warmer ([33]) and likely suffer more mechanical disturbances linked to management ([34], and personal observations) than rural ponds, and both factors might select for a faster life style (Brans et al. in revision). The impact of temperature might be both direct as well as indirect, e.g. through increased activity of invertebrate predators or changed biotic interactions (competition, parasitism). While urbanisation potentially involves many environmental stressors, including pollution, an extensive field study on 81 ponds in the study region revealed only weak differences in nutrient concentrations (slightly lower nitrogen concentrations in urban compared to rural ponds; Gianuca et al 2018), no significant differences in food resources (chlorophyll a concentrations quantifying phytoplankton abundances; Gianuca et al 2018), and no significant differences between urban and rural ponds in overall metal contamination (26 metals) and pesticide concentrations(21 pesticides) between urban and rural ponds (Engelen, PhD). This suggests that differences in water quality or food resources are likely not a recurrent selection factor differentiating rural and urban ponds, although we want to formulate this carefully, as these environmental data were collected during a single snapshot sampling campaign.

The genetic differences we observed between urban and rural populations most likely reflect adaptive evolution to cope with the higher temperatures and stress levels associated with

anthropogenic disturbances in urban environments. As we did not test for fitness differences between urban and rural populations, non-adaptive evolutionary processes can in principle not be ruled out as drivers of the observed genetic differentiation. Yet, given that we selected populations across multiple urbanisation gradients (multiple cities), genetic drift is unlikely to be the driving force of the observed genetic differentiation, as genetic drift has no direction and is thus unlikely to result in the highly repeatable pattern we observed, where city populations behave differently from rural ones irrespective of the region from which they were collected. The significant effect of genotype, nested within population, that we observe furthermore indicates the presence of evolutionary potential for four out of seven traits measured. Finally, the broad set of responsive traits (physiology, life history and heat tolerance) observed in this study and previous work (Brans et al. 2017, in revision), and the fact that heat tolerance has a direct fitness link to temperature differences, provides further evidence for adaptive evolution as a driver of the observed genetic differentiation between urban and rural populations.

Limitations and future directions

We show that urban *Daphnia* populations differ genetically from rural ones for a suit of traits linked to energy metabolism and stress physiology, and that these differences are associated with divergence in mean trait values rather than in trait plasticity. In addition, we show that within the set of urban genotypes there is an overarching pace-of-life syndrome linking both life history and stress physiology. Strikingly, while urban populations also differ in both life history and stress physiology from rural populations, the POLS structure along the urbanisation gradient is different from that within urban populations. Future work on the mechanistic underpinning of the syndromes might reveal why the structure is so different among and within sets of genotypes. Our study involved urban and rural populations sampled across different urbanization gradients in Belgium. Future research across cities on a subcontinental (e.g. European) or global scale will reveal whether our observations can be generalized at these larger spatial scales [x]. Thirdly, and also in support of such a global analysis, it would be a major step forward if genomic signatures of urban evolution could be detected in multiple organisms, such that patterns can be compared

across regions and taxa [x]. While common garden experiments as used in the present study provide strong evidence for genetic differentiation in ecologically relevant traits, they are labor-intensive, which limits their application to large-scale survey studies. In addition, there is a need to quantify the consequences of urban evolution for ecosystem functioning and services to society, such as pollination, pest control, and top-down control of primary producers. Eco-evolutionary dynamics at the ecosystem level in cities are poorly understood and we advocate future studies to include a clear effort to assess ecosystem functions along with trait evolution and ecological dynamics. Finally, more intensive screening of ponds and pools might improve our assessment of the specific drivers of the observed trait change, especially if linked to pollution). A possible solution to reach the latter goals is to include schools and citizens in Citizen Science projects, monitoring ponds in a standardized way via

References

1. McKinney ML. 2008. Effects of urbanization on species richness: A review of plants and animals. *Urban Ecosyst.* **11**, 161-176.
2. Donihue, C. M. & Lambert, M. R. 2014. Adaptive evolution in urban ecosystems. *AMBIO,* 44, 194-203.
3. Brans, K. I., Govaert, L., Engelen, J., Gianuca, A. T., Souffreau, C. & De Meester, L. 2017. Eco-evolutionary dynamics in urbanized landscapes: Evolution, species sorting and the change in zooplankton body size along urbanization gradients. *Philos. Trans. R. Soc. Lond. B. Biol. Sci.,. 372.*
4. Johnson, M. T. J. & Munshi-South, J. 2017. Evolution of life in urban environments. *Science,* 358.
5. Hahs, A. K., McDonnell, M. J., McCarthy, M. A., *et al.* 2009. A global synthesis of plant extinction rates in urban areas. *Ecol. Lett.,* 12, 1165-1173.
6. Munshi-South, J., Zolnik, C. P. & Harris, S. E. 2016. Population genomics of the anthropocene: Urbanization is negatively associated with genome-wide variation in white-footed mouse populations. *Evol. Appl.,* 9, 546-564.
7. Parris, K. M. 2016. *Ecology of urban environments,* UK, Wiley-Blackwell Publishing
8. Partecke, J., Schwabl, I. & Gwinner, E. 2006. Stress and the city: Urbanization and its effects on the stress physiology in European blackbirds. *Ecology,* 87, 1945-1952.

9. Bonier, F., Martin, P. R. & Wingfield, J. C. 2007. Urban birds have broader environmental tolerance. *Biol. Lett.,* 3, 670-673.
10. Isaksson, C. 2015. Urbanization, oxidative stress and inflammation: A question of evolving, acclimatizing or coping with urban environmental stress. *Funct. Ecol.,* 29, 913-923.
11. Meillère, A., Brischoux, F., Parenteau, C. & Angelier, F. 2015. Influence of urbanization on body size, condition, and physiology in an urban exploiter: A multi-component approach. *PLoS ONE,* 10, e0135685.
12. Hutton, P. & McGraw, K. J. 2016. Urban impacts on oxidative balance and animal signals. *Front. Ecol. Evol.,* 4.
13. Korsloot, A., van Gestel, C. A. M. & van Straalen, N. M. 2004. *Environmental stress and cellular responses in arthropods,* Boca Raton, FL, CRC Press.
14. Costantini, D. 2014. *Oxidative stress and hormesis in evolutionary ecology and physiology- A marriage between mechanistic and evolutionary approaches,* Heidelberg, Berlin, Springer.
15. Di Giulio, R. T., Washburn, P. C., Wenning, R. J., Winston, G. W. & Jewell, C. S. 1989. Biochemical responses in aquatic animals: A review of determinants of oxidative stress. *Environ. Toxicol. Chem.,* 8, 1103-1123.
16. Janssens, L. & Stoks, R. 2013. Predation risk causes oxidative damage in prey. *Biol. Lett.,* 9.
17. Chainy, G. B. N., Paital, B. & Dandapat, J. 2016. An overview of seasonal changes in oxidative stress and antioxidant defense parameters in some invertebrate and vertebrate species. *Scientifica,* 2016, 6126570.
18. Burraco, P., Díaz-Paniagua, C. & Gomez-Mestre, I. 2017. Different effects of accelerated development and enhanced growth on oxidative stress and telomere shortening in amphibian larvae. *Sci. Rep.,* 7, 7494.
19. Hoffman, D. J. 2002. Role of selenium toxicity and oxidative stress in aquatic birds. *Aquat. Toxicol.,* 57, 11-26.
20. Demirel, R., Mollaoğlu, H., Yeşilyurt, H., *et al.* 2009. Noise induces oxidative stress in rats. *Eur. J. Gen. Med. ,* 6, 20-24.
21. Isaksson, C. 2010. Pollution and its impact on wild animals: A meta-analysis on oxidative stress. *EcoHealth,* 7, 342-350.
22. Sniegula, S., Janssens, L. & Stoks, R. 2017. Integrating multiple stressors across life stages and latitudes: Combined and delayed effects of an egg heat wave and larval pesticide exposure in a damselfly. *Aquat. Toxicol.,* 186, 113-122.
23. Møller, A. P., Erritzøe, J. & Karadas, F. 2010. Levels of antioxidants in rural and urban birds and their consequences. *Oecologia,* 163, 35-45.
24. Costantini, D., Greives, T. J., Hau, M. & Partecke, J. 2014. Does urban life change blood oxidative status in birds? *J. Exp. Biol.,* 217, 2994-2997.
25. Falfushinska, H. I., Romanchuk, L. D. & Stolyar, O. B. 2008. Different responses of biochemical markers in frogs (*rana ridibunda*) from urban and rural wetlands to the effect of carbamate fungicide. *Comp. Biochem. Physiol. C. Toxicol. Pharmaco.l,* 148, 223-229.
26. Giraudeau, M. & McGraw, K. J. 2014. Physiological correlates of urbanization in a desert songbird. *Integr. Comp. Biol.,* 54, 622-632.
27. Herrera-Dueñas, A., Pineda, J., Antonio, M. T. & Aguirre, J. I. 2014. Oxidative stress of house sparrow as bioindicator of urban pollution. *Ecol. Ind.,* 42, 6-9.
28. Herrera-Dueñas, A., Pineda-Pampliega, J., Antonio-García, M. T. & Aguirre, J. I. 2017. The influence of urban environments on oxidative stress balance: A case study on the house sparrow in the Iberian peninsula. *Front. Ecol. Evol.,* 5.

29. Salmón, P., Stroh, E., Herrera-Dueñas, A., von Post, M. & Isaksson, C. 2018. Oxidative stress in birds along a nox and urbanisation gradient: An interspecific approach. *Sci. Total Environ.,* 622–623, 635-643.
30. Casasole, G., Raap, T., Costantini, D., *et al.* 2017. Neither artificial light at night, anthropogenic noise nor distance from roads are associated with oxidative status of nestlings in an urban population of songbirds. *Com. Biochem. Physiol . A Mol. Integr. Physiol,* 210, 14-21.
31. Ravaschiere, A., Cutler, C., Edleson, K., *et al.* 2017. Quantification of Heat Shock protein 70 and acetylcholinesterase over a time course suggests environmental adaptation in a foundational molluscan species. *Ecotoxicol. Environ. Saf.,* 142, 222-229.
32. Sokolova, I. M. 2013. Energy-limited tolerance to stress as a conceptual framework to integrate the effects of multiple stressors. *Integr. Comp. Biology,* 53, 597-608..
33. Brans, K. I., Engelen, J. M. T., Souffreau, C., & De Meester, L. (2018). Urban hot-tubs: Urban hot-tubs: Local urbanization has profound effects on average and extreme temperatures in ponds. *Landsc. Urban Plan.,* 176, 22-29..
34. Hassall, C. 2014. The ecology and biodiversity of urban ponds. *Wiley Interdisciplinary Reviews: Water,* 1**,** 187-206.Jones, D. K., Mattes, B. M., Hintz, W. D., *et al.* 2017. Investigation of road salts and biotic stressors on freshwater wetland communities. *Environ. Poll.,* 221, 159-167.
35. Crago, J., Corsi, S. R., Weber, D., Bannerman, R. & Klaper, R. 2011. Linking biomarkers to reproductive success of caged fathead minnows in streams with increasing urbanization. *Chemosphere,* 82, 1669-1674.
36. Réale, D., Garant, D., Humphries, M. M., Bergeron, P., Careau, V. & Montiglio, P.-O. 2010. Personality and the emergence of the pace-of-life syndrome concept at the population level. *Philos. Trans. R. Soc. Lond. B. Biol. Sci.,* 365, 4051-4063.
37. Tüzün, N., Müller, S., Koch, K. & Stoks, R. 2017a. Pesticide-induced changes in personality depend on the urbanization level. *Anim. Behav.,* 134, 45-55.
38. Niemelä, P. T., Dingemanse, N. J., Alioravainen, N., Vainikka, A. & Kortet, R. 2013. Personality pace-of-life hypothesis: Testing genetic associations among personality and life history. *Behav. Ecol.,* 24, 935-941.
39. Debecker, S., Sanmartín-Villar, I., de Guinea-Luengo, M., Cordero-Rivera, A. & Stoks, R. 2016. Integrating the pace-of-life syndrome across species, sexes and individuals: Covariation of life history and personality under pesticide exposure. *J. Anim. Ecol.,* 85, 726-738.
40. Brans, K. I., Jansen, M., Vanoverbeke, J., Tüzün, N., Stoks, R. & De Meester, L. 2017. The heat is on: Genetic adaptation to urbanization mediated by thermal tolerance and body size. *Glob.Change. Biol.,* 23, 5218-5227.
41. Dumont, H. J., Van de Velde, I. & Dumont, S. 1975. The dry weight estimate of biomass in a selection of Cladocera, Copepoda and Rotifera from the plankton, periphyton and benthos of continental waters. *Oecologia,* 19, 75-97.
42. Bradford, M. M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Analyt. Biochem.,* 72, 248-254.
43. Bligh, E. G. & Dyer, W. J. 1959. A rapid method of total lipid extraction and purification. *Can. J. Biochem. Phys.,* 37, 911-917.
44. Stoks, R., Block, M. D. & McPeek, M. A. 2006. Physiological costs of compensatory growth in a damselfly. *Ecology,* 87, 1566-1574.

45. De Block, M. & Stoks, R. 2008. Compensatory growth and oxidative stress in a damselfly. *Proc. R. Soc. B.,* 275, 781-785.
46. McLoughlin, N., Yin, D., Maltby, L., Wood, R. M. & Yu, H. 2000. Evaluation of sensitivity and specificity of two crustacean biochemical biomarkers. *Environ. Toxicol. Chem.,* 19, 2085-2092.
47. Grotto, D., Maria, L. S., Valentini, J., *et al.* 2009. Importance of the lipid peroxidation biomarkers and methodological aspects for malondialdehyde quantification. *Química Nova,* 32, 169-174.
48. R Development Core Team 2016. R: A language and environment for statistical computing. Vienna, Austria: R Foundation for Statistical Computing.
49. Grace, J. B. 2006. *Structural equation modeling and natural systems,* Cambridge, UK, Cambridge University Press.
50. Dingemanse, N. J., Dochtermann, N. & Wright, J. 2010. A method for exploring the structure of behavioural syndromes to allow formal comparison within and between data sets. *Anim. Behav.,* 79, 439-450.
51. Shipley, B. 2009. Confirmatory path analysis in a generalized multilevel context. *Ecology,* 90, 363-368.
52. Zar, J. 1999. *Biostatistical analysis,* Upper Saddle River, New Jersey, USA, Prentice Hall.
53. Rosseel, Y. 2012. Lavaan: An R package for structural equation modeling. *2012,* 48, 36.
54. Harris, S. E. & Munshi-South, J. 2017. Signatures of positive selection and local adaptation to urbanization in white-footed mice *Peromyscus leucopus* . *Mol Ecol.*,26:6336–6350.
55. Mueller, J. C., Partecke, J., Hatchwell, B. J., Gaston, K. J. & Evans, K. L. 2013. Candidate gene polymorphisms for behavioural adaptations during urbanization in blackbirds. *Mol. Ecol.,* 22, 3629-3637.
56. De Coen, W. M. & Janssen, C. R. 2003. The missing biomarker link: Relationships between effects on the cellular energy allocation biomarker of toxicant-stressed *Daphnia magna* and corresponding population characteristics. *Environ.Toxicol. Chem.,* 22, 1632-1641.
57. Barata, C., Markich, S. J., Baird, D. J., Taylor, G. & Soares, A. M. V. M. 2002. Genetic variability in sublethal tolerance to mixtures of cadmium and zinc in clones of *Daphnia magna* Straus. *Aquat. Toxicol.,* 60, 85-99.
58. Jemec, A., Tišler, T., Erjavec, B. & Pintar, A. 2012. Antioxidant responses and whole-organism changes in *Daphnia magna* acutely and chronically exposed to endocrine disruptor bisphenol a. *Ecotoxicol. Environ. Saf.,* 86, 213-218.
59. Coldsnow, K. D., Mattes, B. M., Hintz, W. D. & Relyea, R. A. 2017. Rapid evolution of tolerance to road salt in zooplankton. *Environ. Poll.,* 222, 367-373.
60. Sarnelle, O. & Wilson, A. E. 2005. Local adaptation of *Daphnia pulicaria* to toxic cyanobacteria. *Limnol. Oceanogr.,* 50, 1565-1570.
61. Zhang, C., Jansen, M., De Meester, L. & Stoks, R. 2016. Energy storage and fecundity explain deviations from ecological stoichiometry predictions under global warming and size-selective predation. *J. Anim. Ecol.,* 85, 1431-1441.
62. Cheptou, P.-O., Carrue, O., Rouifed, S. & Cantarel, A. 2008. Rapid evolution of seed dispersal in an urban environment in the weed *Crepis sancta*. *PNAS,* 105, 3796-3799.
63. Thompson, K. A., Renaudin, M. & Johnson, M. T. J. 2016. Urbanization drives the evolution of parallel clines in plant populations. *Proc. Roy. Soc. B Biol. Sci.,* 283.
64. Diamond, S. E., Chick, L., Perez, A., Strickler, S. A. & Martin, R. A. 2017. Rapid evolution of ant thermal tolerance across an urban-rural temperature cline. *Biol. J. Linn. Soc. Lond.,* 121, 248-257.

65. Ricklefs, R. E. & Wikelski, M. 2002. The physiology/life-history nexus. *TREE*, 17, 462-468.
66. Killen, S. S., Marras, S., Metcalfe, N. B., McKenzie, D. J. & Domenici, P. 2013. Environmental stressors alter relationships between physiology and behaviour. *TREE*, 28, 651-658.
67. Charmantier, A., Demeyrier, V., Lambrechts, M., Perret, S. & Grégoire, A. 2017. Urbanization is associated with divergence in pace-of-life in great tits. *Front. Ecol. Evol.*, 5.
68. Bell, A. M. & Sih, A. 2007. Exposure to predation generates personality in threespined sticklebacks (*Gasterosteus aculeatus*). *Ecol. Lett.*, 10, 828-834.
69. Pauwels, K., Stoks, R. & De Meester, L. 2010. Enhanced anti-predator defense in the presence of food stress in the water flea *Daphnia magna*. *Funct. Ecol.*, 24, 322-329.
70. Rauw, W. M. 2012. Immune response from a resource allocation perspective. *Fron. Gen.*, 3, 267.
71. Angilletta, M. J., Jr. 2009. *Thermal adaptation. A theoretical and empirical synthesis.*, Oxford, Oxford University Press.

Competing Interests

The authors declare no competing interests.

Acknowledgements

We thank Ria Van Houdt for her contribution to the sample processing. We thank Carla Denis and Melissa Schepens for culturing the algae and contributing to the microsatellite analysis. We thank Marc Johnson, Ruth Rivkin, James Santangelo, and three anonymous reviewers for their constructive feedback and comments on the manuscript. We thank the IAP SPEEDY consortium for providing logistic support and Belspo for funding (IAP SPEEDY). This work was also financially supported by KU Leuven Research Council funding PF/2010/07 and C16/17/002. KIB acknowledges a FWO PhD (Aspirant) fellowship.

Author Contributions

KIB and LDM designed the experiment. KIB performed the experiment and performed the statistical analyses with input of LDM and RS. KIB wrote the first draft of the manuscript. LDM and RS contributed to the subsequent versions of the manuscript.

Data accessibility

Data are available online (Dryad Repository) after editorial decision.

633

Table 1.

	Urbanisation				Temperature				Urbanisation x Temperature				Clone (random)	
Manova	**F**	**ndf**	**Pillai**	**P**	**F**	**ndf**	**Pillai**	**P**	**F**	**ndf**	**Pillai**	**P**		
All traits	7.820	7	0.543	< **0.001**	1.230	7	0.158	0.306	1.022	7	0.134	0.429	*Included as random error term*	
Univariate anovas	**F**	**ndf**	**ddf**	**P**	**F**	**ndf**	**ddf**	**P**	**F**	**ndf**	**ddf**	**P**	χ^2	**P**
Total protein content	16.265	1	10.090	**0.002**	5.486	1	353.07	**0.020**	0.174	1	353.06	0.677	2.648	0.050·
Total fat content	14.671	1	9.68	**0.003**	2.985	1	352.40	0.085·	0.038	1	352.40	0.846	8.102	**0.002**
Total sugar content	23.0736	1	9.980	< **0.001**	1.166	1	351.18	0.281	0.068	1	351.28	0.794	9.5027	**0.001**
MDA (lipid peroxidation)	0.045	1	10.68	0.835	0.579	1	355.25	0.447	0.238	1	354.37	0.626	0.106	0.372
SOD	8.808	1	9.85	**0.014**	12.779	1	358.84	< **0.001**	0.001	1	258.84	0.973	2.598	0.054·
CAT	0.435	1	10.01	0.525	29.439	1	356.850	< **0.001**	0.077	1	357.080	0.781	3.223	**0.037**
GST	7.282	1	9.79	**0.023**	0.141	1	357.89	0.707	1.419	1	358.01	0.243	5.447	**0.010**

Figures

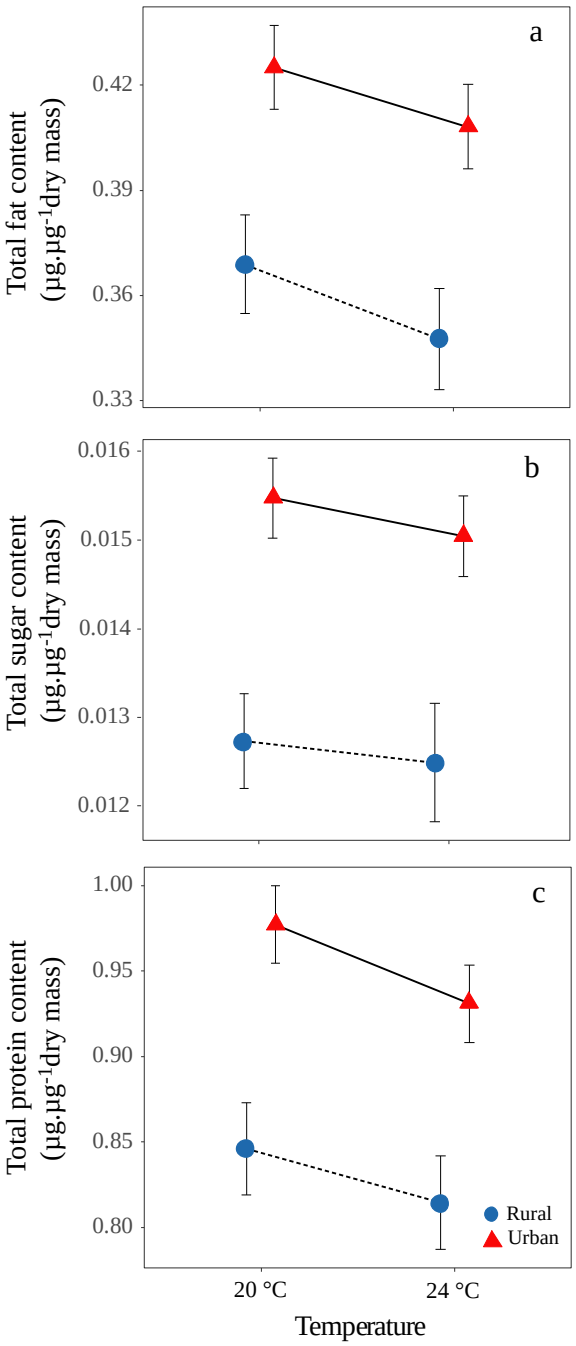

Figure 1.

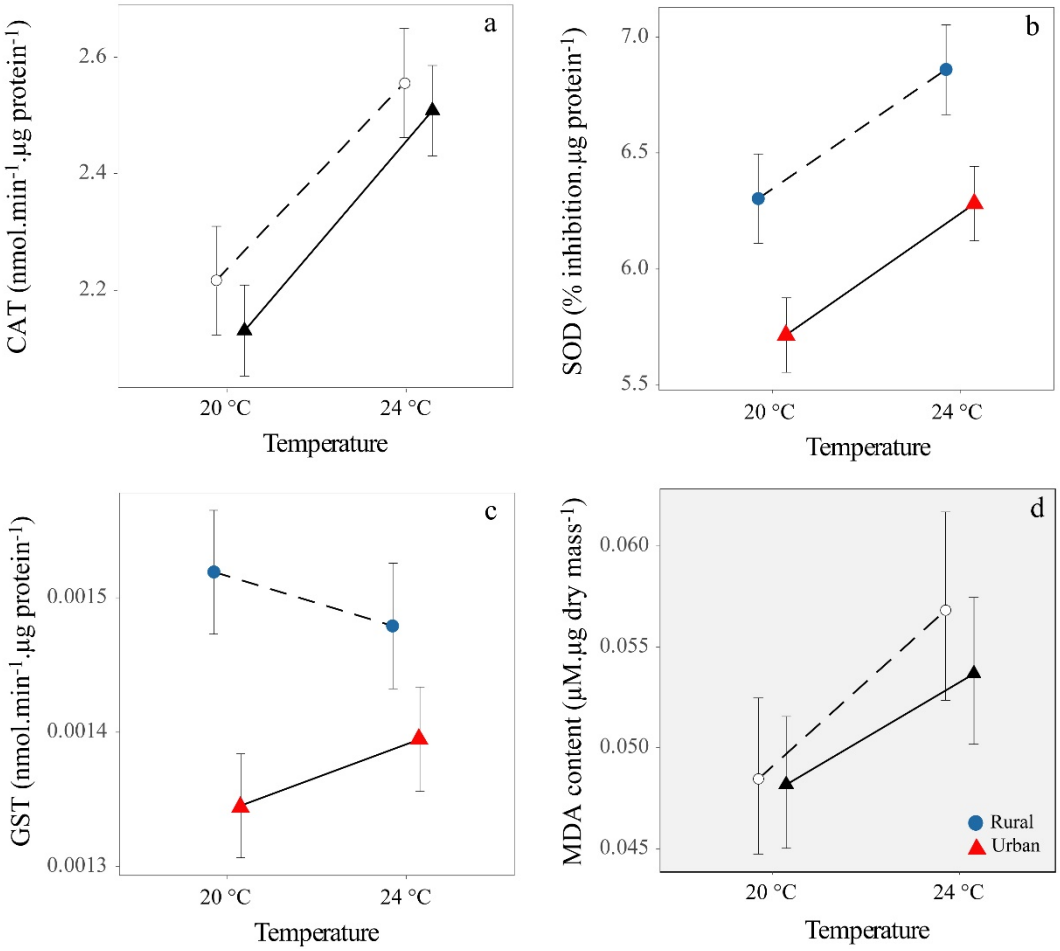
a
CAT (nmol.min-1.µg protein-1)
2.6
2.4
2.2
20 °C
24 °C
Temperature
b
SOD (% inhibition.µg protein-1)
7.0
6.5
6.0
5.5
20 °C
24 °C
Temperature
c
GST (nmol.min-1.µg protein-1)
0.0015
0.0014
0.0013
20 °C
24 °C
Temperature
d
MDA content (µM.µg dry mass-1)
0.060
0.055
0.050
0.045
Rural
Urban
20 °C
24 °C
Temperature

Figure 2.

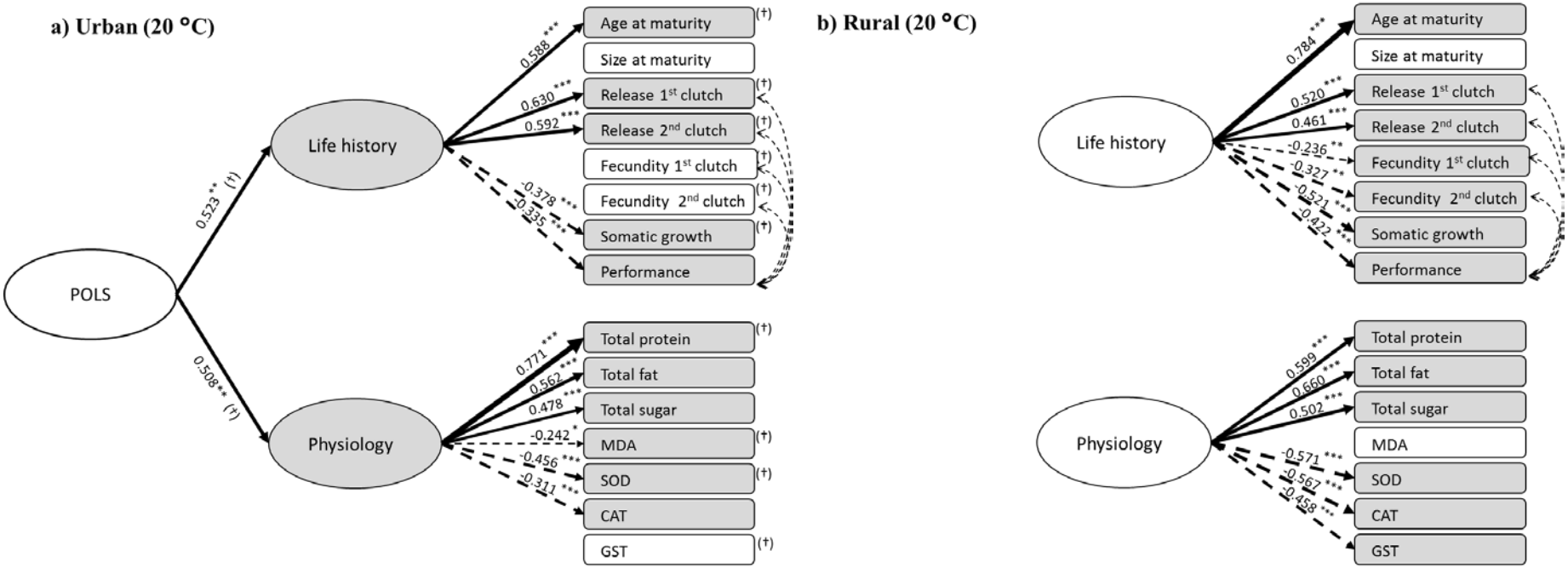
a) Urban (20 °C)
POLS
Life history
Physiology
0.523 ** (†)
0.508** (†)
0.588 ***
0.630 ***
0.592 ***
-0.378 ***
-0.335 ***
0.771 ***
0.562 ***
0.478 ***
-0.242 *
-0.456 ***
-0.311 ***
Age at maturity
Size at maturity
Release 1st clutch
Release 2nd clutch
Fecundity 1st clutch
Fecundity 2nd clutch
Somatic growth
Performance
Total protein
Total fat
Total sugar
MDA
SOD
CAT
GST
(†)
b) Rural (20 °C)
Life history
Physiology
0.784 ***
0.520 ***
0.461 ***
-0.236 **
-0.327 **
-0.521 ***
-0.422 ***
0.599 ***
0.660 ***
0.502 ***
-0.571 ***
-0.567 ***
-0.458 ***
Age at maturity
Size at maturity
Release 1st clutch
Release 2nd clutch
Fecundity 1st clutch
Fecundity 2nd clutch
Somatic growth
Performance
Total protein
Total fat
Total sugar
MDA
SOD
CAT
GST

Figure 3.

Table captions

Table 1. Multivariate and univariate statistical results. Results of the MANOVA and univariate linear mixed-effect models, testing for the effect of urbanisation level, treatment, and their interaction on a set of seven physiological parameters linked to energy metabolism (total protein, fat, and sugar content) and oxidative stress (antioxidant enzymes superoxide dismutase (SOD), glutathione-S-transferase (GST), and catalase (CAT); oxidative damage: lipid peroxidation (MDA)), measured in a set of urban and rural *Daphnia magna* genotypes grown under common garden conditions. Total fat content was used as a covariate in the MDA model. Significant results ($p < 0.05$) are given in bold or indicated with "·" ($p < 0.1$). Numerator and denominator degrees of freedom are given by ndf and ddf respectively.

Figure captions

Figure 1. Energy storage compounds. Mean ± 1 SE fat (a), sugar (b), and protein (c) content for urban (triangles) and rural (circles) animals reared at 20 °C and 24 °C. Significant differences between urban and rural animals are given by colored symbols (urban: red; rural: blue).

Figure 2. Antioxidant enzymes (a-c, white) and oxidative damage (d, grey). Mean ± 1 SE CAT activity (a), SOD activity (b), GST activity (c), and lipid peroxidation, measured as MDA (d) for urban (triangles) and rural (circles) animals reared at 20 °C and 24 °C. Significant differences between urban and rural animals are given by colored symbols (urban: red; rural: blue). Non-significant differences for the effect of urbanisation are depicted in black/white.

Figure 3. Structural equation models. Path models showing the covariation pattern of life history and physiological traits (i.e. measured traits, boxes) within a life history, physiology, and overarching pace-of-life syndrome (POLS) (all modeled as latent variable, ovals) in a) urban and b) rural genotype sets of *Daphnia magna* when reared at 20 °C. Differences in covariation structures between the two groups reflect genetic differentiation. Only paths significantly contributing to syndrome structure (i.e. significantly different from 0) are depicted (associated traits are in grey colored boxes), and accompanied with path coefficients. Significance levels of path coefficients are depicted as *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$. Path coefficients indicate the change (in SD units) of traits predicted to occur based on a 1 SD change in the underlying syndrome structure. Arrow thickness (dashed arrows: negative correlations, full arrows: positive correlations) is proportional to the strength of the path loading. Path loadings significantly differing between

urban and rural genotype sets (either because of their significant contribution to a syndrome in one group, but not in the other, or because of a significant difference in direction or size of path loadings that significantly contribute to syndrome structures in both groups) are indicated with † ($p < 0.05$ after Bonferroni correction). Dashed double-headed arrows indicate correlated errors.