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City life on fast lanes: urbanization induces an evolutionary shift towards a faster life style in the water flea *Daphnia*

Running title: Urbanization drives pace-of-life evolution

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Abstract

1. Multiple species show significant trait shifts in response to urbanization. Yet, the impact of anthropogenic warming linked to the urban heat island effect is remarkably understudied. Additionally the relative contributions of phenotypic plasticity and genetic change underlying trait shifts in response to urbanization are poorly known.
2. A common garden study with the water flea *Daphnia magna* revealed that both genetic differentiation in response to urbanization and phenotypic plasticity in response to higher rearing temperature (24 °C) induced significant parallel multivariate shifts in life history strategy along the slow-fast pace-of-life axis.
3. Urban animals and animals reared at higher temperatures are characterized by fast maturation, early release of progeny, a smaller size at maturity, increased fecundity, and higher performance (given by maximal population growth rate *r*) compared to genotypes isolated from rural ponds and animals reared at lower temperatures respectively.
4. Evolution in response to urbanization accounted for 30% of the total observed shift in life history and caused a significant change in mean trait values, while plasticity responses to experimental warming were unaltered between urban and rural populations. The total trait change achieved through both plasticity and evolution ranged from 8 to 56% depending on the trait.
5. Our results provide clear evidence for evolution underlying an increase in pace-of-life of populations in response to urbanization. Given the pivotal role of *Daphnia* in aquatic ecosystems, this shift potentially feeds back to population structure, top-down control of algae, and food web dynamics in urban freshwater ecosystems. Additionally, we argue that adaptation to urban heat islands might render these populations pre-adapted in a context of future climate change.

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Key words: eco-evolutionary partitioning, *Daphnia magna*, pace-of-life, performance, phenotypic plasticity, Phenotypic Trajectory Analysis, urban ecology and evolution, urban heat islands.

INTRODUCTION

Urban environments are increasingly dominant in contemporary landscapes, and represent an exceptional combination of different dynamic abiotic and biotic disturbance gradients: cities are more chemically and physically polluted, eutrophied, fragmented, noisy, warmer, artificially lighted, and have a higher occurrence of exotic species compared to rural and natural areas (Gilbert, 1989; Kaye et al., 2006; Grimm et al., 2008; Ramalho & Hobbs, 2012; Douglas & James, 2015; Knop et al., 2017). These profound and complex environmental changes induce fast and pronounced ecological and evolutionary changes in many species (Cheptou et al., 2008; Donihue & Lambert, 2014; Parris, 2016; Alberti et al., 2017; Brans et al., 2017a,b). As a consequence of the earlier onset of growing seasons in urban areas, many plant species show changes in flowering time, size, and seed production (Neil & Wu, 2006; Palma et al., 2016), while birds shift egg laying date and mating behavior (Slabbekoorn et al., 2013; Cardoso, 2014; Møller et al., 2015). Most studies, however, report on field observations, and thus confound genetic differences and phenotypic plasticity responses. It has been questioned whether phenotypic plasticity is sufficient to assure long-term population persistence of species when exposed to strong environmental disturbances (Fuller et al., 2010; Duputié et al., 2015; Gunderson & Stillman, 2015; Diamond & Martin, 2017). As more studies engage in common garden approaches, the evidence of genetic differentiation between rural and urban populations for physiological, morphological, life history, and behavioral traits is rapidly growing (Cheptou et al., 2008; San Martin Y Gomez & Van Dyck, 2012; Yakub & Tiffin, 2016; Brans et al., 2017a, 2017b). A substantial number of studies

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report clear signals of urban adaptations using percentage built-up area or impervious surfaces in the surroundings of the habitat, or distance from the city center. These metrics serve as a proxy for urbanization, integrating all environmental disturbances associated with city environments. Urbanization is a complex mixture of environmental disturbances and often cannot be traced back to one specific environmental axis. As not all abiotic and biotic changes associated with urbanization can be properly quantified (e.g. some pollution events are short-lived), the approach of using percentage built-up area or impervious surfaces as a proxy is a suitable and straightforward method to assess adaptations in response to the multitude of stressors organisms are exposed to when living in cities. For example, Sprau et al. (2016) found that the phenotypic variation in great tits (*Parus major*) inhabiting urban and rural plots, known to differ in temperature, humidity, and light conditions, were best explained by the simple dichotomy 'urban/rural' in comparison with using the specific environmental variables assessed in all plots.

A well-known extension to classical life history theory (Roff, 1992, 2002; Stearns, 1992) is the 'pace-of-life' (POL) concept (Saether, 1988; Promislow & Harvey, 1990; Ricklefs, 2000; Wiersma et al., 2007), in which individuals, populations, and species are ranked along a 'slow-to-fast' pace-of-life axis reflecting covariation among life history traits (Réale et al., 2010; Debecker et al., 2016). 'Fast living' organisms are characterized by rapid development and maturation, small body size, high fecundity, and often reduced lifespan. 'Slow living' organisms fall on the opposite end of the continuum. This concept is closely linked to the physiological processes that support it (i.e. the life history/physiology-nexus; Ricklefs & Wikelski, 2002; Londoño et al., 2015), and other organismal traits, together synthesized in the Pace-of-life Syndrome (POLS) hypothesis. This POLS hypothesis states that physiological, behavioral, and immunological characteristics of closely related species or populations experiencing contrasting environments covary with specific life history traits

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evolved in these contrasting environments (Brodin & Johansson, 2004; Réale et al., 2010; Debecker et al., 2016; Monceau et al., 2017; Segev et al., 2017). The POLS hypothesis has been studied in a broad range of species, from invertebrates (Van Voorhies & Ward, 1999; Niemelä et al., 2013; Debecker et al., 2016) to birds (Wikelski et al., 2003; Londoño et al., 2015) and mammals (Oli, 2004; Nilsen et al., 2009). Yet most studies on pace-of-life divergence focus on between-species level differences (e.g Debecker et al., 2016.) or shifts between populations along large scale climatic gradients (e.g. across latitudes; Londoño et al., 2015). Evidence on pace-of-life divergence along small scale climatic gradients is remarkably understudied.

Anthropogenic activity, energy consumption, and the radiation of heat by buildings at night cause cities worldwide to heat up, a general and, more important, re-occurring phenomenon known as the 'urban heat island effect' (Oke, 1973, 1982; Santamouris, 2007; Ward et al., 2016). For aquatic ecosystems such as freshwater ponds and pools, a similar urban heat island effect (i.e. an urban 'hot-tub effect') was recently reported, with city ponds having higher mean, minimum, and maximum temperatures (Brans et al., in press; see also Fig. S1, Supplementary Information). In this study, it was found that the average summer mean and maximum temperature differences between city and rural ponds reached 3.04 °C and 3.69 °C, respectively. We recently reported a genetic increase in heat tolerance in urban compared to rural populations of the water flea *Daphnia magna* (Brans et al., 2017b). Such an increase in heat tolerance was also reported in a study on urban thermal adaptation in a terrestrial organism, acorn ants (Diamond et al., 2017). Yet, warming is also intrinsically related to key life history traits such as growth rate, fecundity, and survival (Angilletta, 2009). In addition, the urbanization gradient might also include other stressors (e.g. increased levels of disturbance such as those linked to the management of the water bodies) that might select for differences in life histories. Although recent studies explored phenotypic life

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history shifts in city dwellers (Sprau et al., 2016; Charmantier et al., 2017; Sol et al., 2018), surprisingly little is known on genetic differences underlying pace-of-life in response to urbanization. Especially ectotherm animals, whose metabolic rates and growth are substantially driven by environmental temperatures, will be prone to respond to higher average and maximum temperatures, and increased temperature fluctuations (Angilletta et al., 2004; Deutsch et al., 2008; Daufresne et al., 2009; Hoffmann et al., 2013). It is not straightforward to predict the direction of evolutionary responses to urban heat islands and other urbanization-linked stressors. In ectotherm species, one might predict that warming in cities might induce evolution of a fast life cycle involving small individuals that develop fast and produce many, smaller eggs. This prediction is in line with the temperature-size rule (Atkinson, 1994; Forster et al., 2012; Hoefnagel & Verberk, 2015) and complements the earlier observation that small organisms have a higher thermal tolerance, as was also reported for *Daphnia* (Geerts et al., 2015; Brans et al., 2017b). The occurrence of disturbances linked to management (e.g. clearance of litter layer on the bottom of the pond and addition of water; Hassall, 2014; personal observations) and extreme temperature events in cities (Ward et al., 2016; Wouters et al., 2017; Brans et al., in press) might also select for a fast life cycle (Evans et al., 2010; Atwell et al., 2012; Charmantier et al., 2017). Alternatively, the limited availability of oxygen in aquatic systems and the increased oxygen demand at higher temperatures (Bozinovic & Pörtner, 2015; Chown et al., 2015; Horne et al., 2015) might lead to the expectation of lower metabolic rates and thus a slower life style in urban compared to rural populations of ectotherms (Angilletta et al., 2004; Tüzün et al., 2017). One might also expect countergradient responses, such as those recently shown by Tüzün et al. (2017) in damselflies, where urban populations genetically grow slower (at all tested rearing temperatures in the laboratory) compared to rural populations as a likely response to longer

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growing seasons in urban ponds (Brans et al., in press), that result in relaxed time constraints for urban animals.

Here we test whether urban populations of *Daphnia magna*, a key grazer in the pelagic of shallow, eutrophic ponds and lakes, are genetically differentiated from rural populations for their life history traits, and whether these changes are situated along the slow-fast life history axis, in line with the pace-of-life hypothesis (Réale et al., 2010). We performed a common garden experiment using *D. magna* genotypes originating from a total of 13 populations along strong urbanization gradients. To quantify the relative importance of evolution of mean trait values, plasticity to warming, and evolution of thermal plasticity, animals were reared at two temperatures (20 °C and 24 °C), approximating the temperature differences currently observed between rural and urban ponds in Flanders (Northern Belgium) during spring and summer (Brans et al., 2017a; Brans et al., in press, Fig. S1 Supplementary Information,). We quantified a set of eight life history traits and assessed genetic differentiation (rural vs. urban), plastic responses for the range 20- 24 °C, and interactions between evolution and plasticity across this temperature range (urbanization-dependent thermal plasticity). We address trait divergence from both a univariate and multivariate approach, testing the hypothesis that urban populations have genetically adapted to live 'faster', i.e. have a higher growth rate, mature earlier, and release their young faster. Although a fast life history strategy might translate in the production of more offspring, rapid development and maturation at a smaller size potentially comes at a cost of the production of smaller clutches. In summary, this study is the first to tap in to evolutionary pace-of-life divergence along urbanization gradients and additionally aims to quantify the relative contribution of evolution, phenotypic plasticity, and evolution of plasticity to the total observed intraspecific phenotypic trait changes observed between rural and urban populations. Our common garden approach does go beyond showing phenotypic responses,

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as it allows disentangling the importance of phenotypic plasticity and evolutionary divergence to the observed phenotypic trait shifts in response to urbanization.

MATERIAL AND METHODS

Study organism and urbanization gradient

The populations and genotypes used in the present study are identical to those in our earlier study reporting evolution of increased thermal tolerance (Brans et al., 2017b). Thirteen populations of the water flea *D. magna* were sampled in Flanders (Northern Belgium) in the summer of 2013, and categorized according to the surrounding percentage built-up area (3200 m radius, reflecting regional levels of urbanization; see Fig. S2 for correlations with measurements of %BA at smaller spatial scales) into urban (> 10%) and rural (< 5%). %BA refers to the percentage surface covered by buildings and does not include roads and parking lots. As a result, areas characterized by more than 10% of BA are already substantially urbanized (see Fig. 1). Rural populations were characterized by an additional criterion of minimum 20% of Biologically Valuable Area in the regional surroundings of the pond (3200 m radius). Detailed information on sampling locations, the assessment of urbanization, and pre-experimental culturing procedures can be found in Supplementary Information (Supplementary Information A; Supplementary Information B: Fig. 1, Table S1).

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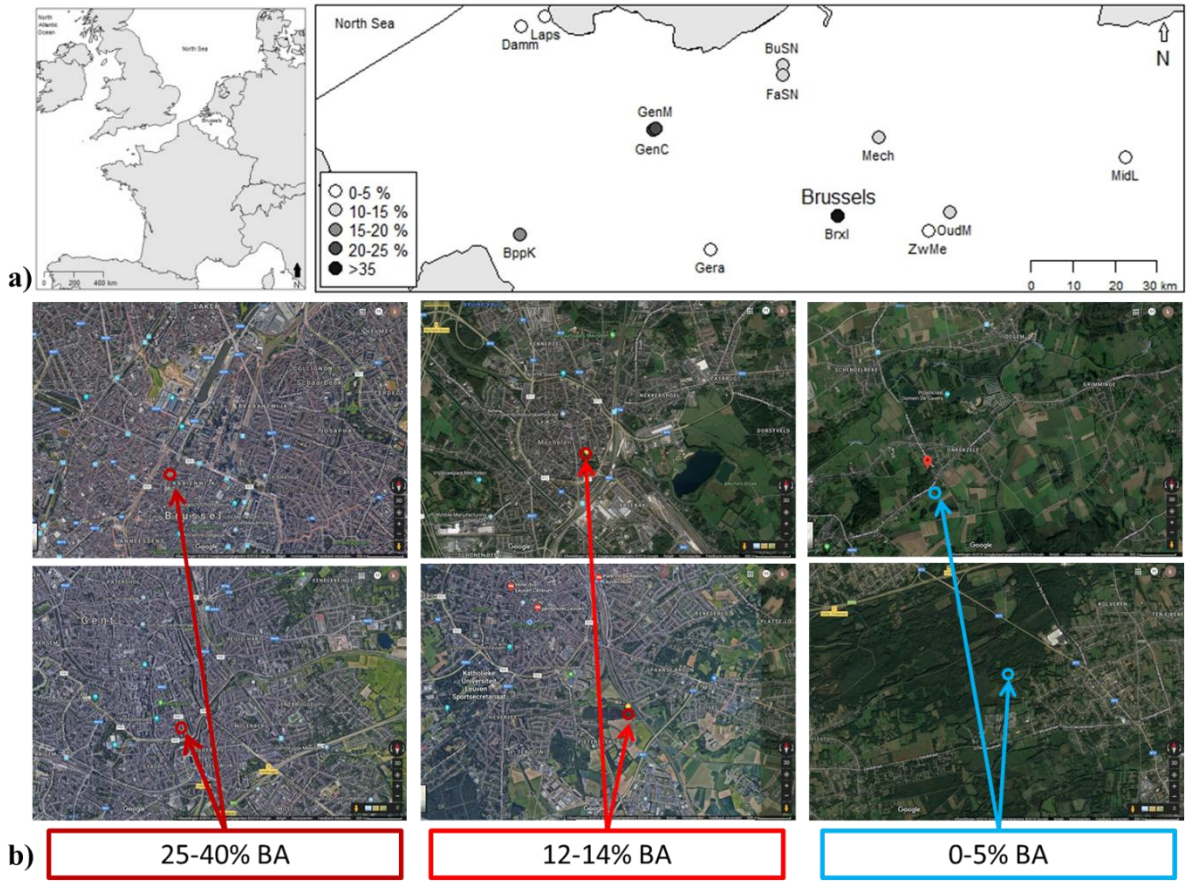

Figure 1. (a) Sampling locations of the *D. magna* study populations in Flanders (Inset: Wider region of Europe, depicting Belgium). Urbanization classes are given according to the percentage built-up area in a radius of 3200 m around each pond. Ponds surrounded with more than 10% of built-up area are considered urban. Rural ponds have an additional criterion of 20% Biologically Valuable area at a radius of 3200 m around the pond. (b) Examples of sampled ponds in urban settings (25-40% BA: upper: city of Brussels; lower: city of Ghent; 12-14%: upper: city of Mechelen; lower: city of Leuven) and in rural settings (category 0-5% BA) to illustrate the point that >10% BA represents highly urbanized settings. The circles represent the sampling locations (ponds). All ponds are located here in a plot of ca. 6 x 4 km here, in which the regional scale of urbanization is in concordance with the 3200 m radius at which %BA was assessed.

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Experimental set-up and response variables

Overall design and response variables

To test for an effect of urbanization level and temperature treatment on *D. magna* life history traits, we carried out a full factorial experiment (design: 13 populations x 6 independent lines x 2 temperatures; 20 °C and 24 °C; *Brxl* only 4 lines; each combination in triplicate except *Brxl*: one replicate; see also Supplementary Information A) resulting in 440 experimental units. After purging maternal and grandmaternal effects (See Supplementary Information A), a cohort of 12 juveniles (< 24h old) of each culture was inoculated in a 500ml jar and assigned to the appropriate temperature treatment (water baths set at 20 °C and 24 °C, 14:10 L:D photoperiod). Medium refreshment and feeding were standardized (see Supplementary Information A). We measured size at birth for three individuals for each experimental cohort. At maturity, six animals per experimental cohort were followed-up to quantify life history traits (the six others were scored for thermal tolerance; see Brans et al., 2017b).

Life history traits

The experimental cohorts (n = 220 per temperature treatment) were monitored on a daily basis and the following life history traits were scored: age and size at maturity, age at release of the first and second clutch, and the number of juveniles in the first and second clutch (number of juveniles/female, to assess fecundity). Size measurements were performed on four animals per cohort (measured from the center of the eye to the base of the tail spine). From these life history measures we estimated two additional life history traits: somatic growth *Gj*, calculated as *Gj = (ln (size at maturity) – ln (size at birth))/age at maturity*, and individual performance *r*, calculated by iteratively solving the Euler equation to 1 ($\sum e^{-rx} l_x m_x = 1$; Stearns, 1992). This latter measure of performance translates into population growth rate assuming no variation between individuals.

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Statistical analysis

All statistical analyses were performed with the R software version 3.3.2 (R Development Core Team, 2016).

MANOVA and univariate mixed-effect models

To test for overall differentiation in life history traits between urban and rural *Daphnia*, and between animals reared at 20 °C versus 24 °C, we performed MANOVA on all response variables scaled to a mean of 0 and standard deviation of 1. To test for differentiation in the different life history traits between urban and rural *Daphnia*, and between animals reared at 20 °C and 24 °C, we subsequently ran separate linear mixed-effect models for each of the eight response traits. For both the MANOVA and the univariate mixed-effect models, we used urbanization, temperature treatment and their interaction term as fixed factors, and population nested in urbanization, and clone nested in population, as random effects. To assess a possible effect of clonal identity (implying genetic variation for a certain trait), we applied model comparison of linear mixed-effect models with and without this random factor. The computation of test statistics, the multivariate and univariate model specifications, assumption verifications, and used R packages are detailed in Supplementary Information A. We additionally tested random slopes models for each linear mixed-effect model to control for any clone or population specific G x E interactions (E: temperature treatment), thus allowing both intercepts and slopes to vary between clones and populations (random factors) within urbanization-level in response to temperature. The results of these models were qualitatively identical to the random intercept models, and AIC score comparisons only indicated a higher preference for the random slopes model in two (age at maturity, release of second clutch) out of eight measured variables. In both cases however, results were the same

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(for release of the second clutch the effect of urbanization changed from marginally significant to significant – $p = 0.014$, see Table 1 of the Results for comparison). We here thus overall report random intercept models.

Principal Component Analysis (PCA) and Phenotypic Trajectory Analysis (PTA)

To complement MANOVA results and visualize the multivariate responses, we conducted PCA (Principal Component Analysis) and PTA (Phenotypic Trajectory Analysis; Collyer & Adams, 2007; Adams & Collyer, 2009) to: (i) detect the principal modes of variation and covariation among the measured life history traits, (ii) better describe and visualize the multivariate phenotypic trajectories of the populations for the studied temperature range (20-24 °C), (iii) test for multivariate convergence or divergence of urban and rural populations within temperature treatments, and (iv) test for the evolution of multivariate reaction norms (Plaistow & Collin, 2014). PTA was performed using the approach described in Collyer & Adams (2007) and Stoks et al. (2016), applying a residual randomization procedure, and including random effects and nested designs (population, nested in urbanization, and clone, nested in population, both as random factors). In short, this method first allows calculating the length (magnitude) and angle (or direction, θ) of multivariate vectors of trait change (here: between temperature treatments) and enables to compare these between groups (Fig. 2a dashed lines). The method can further be extended to test for multivariate convergence or divergence of populations by comparing the orientation of the reaction norms (Dennis et al., 2011) and the Euclidean distances between centroids (Fig. 2b full lines). This approach thus can also be used to assess whether groups are distinct in multivariate trait space, which in the current case tests whether the difference in the trajectory of the multivariate genotypic trait values between urban and rural populations in a certain temperature treatment is significantly

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different from 0 (see 'evolutionary trajectories' Fig. 2b). PTA and PCA procedures are described in detail in Supplementary Information A.

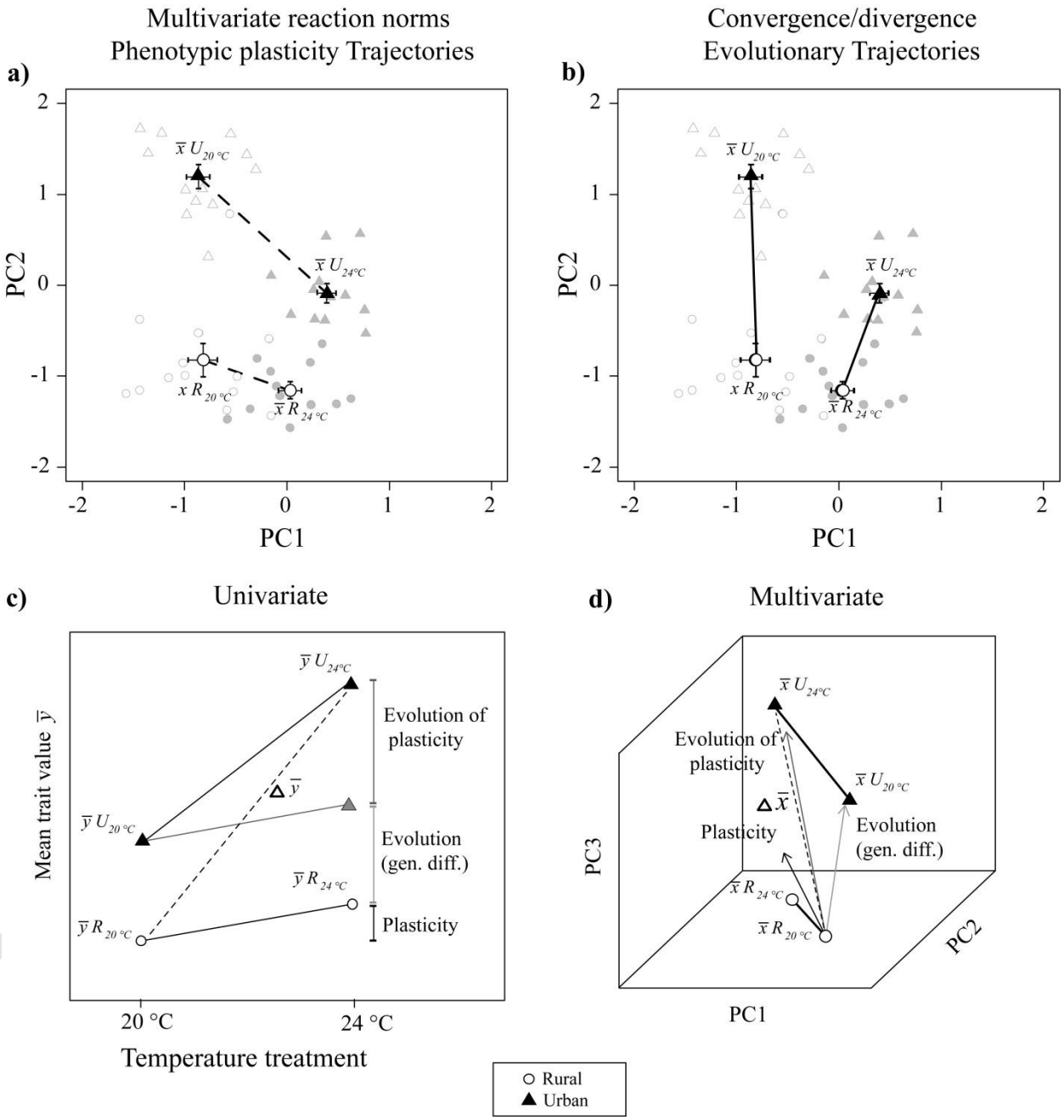

Figure 2. Schematic methodological illustrations. Upper plots: Principal Components Analysis plots schematically illustrating how Phenotypic Trajectory Analysis (PTA) was applied to (a) test for differences in the magnitude and angle of multivariate reaction norms (i.e. 'phenotypic plasticity trajectories') of, for instance, rural (circles) and urban (triangles) populations (small grey symbols here indicate population means, large black symbols are group means), reared at 20 °C (open grey symbols) and 24 °C (filled grey symbols), and (b) test for convergence or divergence and genotypic differentiation (referred to as 'evolutionary trajectories'; symbols are colored likewise); adapted from Stoks et al. (2016).

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Lower plots: Illustrative example adapted from Govaert et al. (2016) of (c) the univariate reaction norm for a certain phenotypic trait Y, with mean trait values for urban (U, black triangles) and rural (R, white circles) *Daphnia* reared at 20 °C and 24 °C (indicated in index), and (d) the multivariate reaction norm for a set of *n* phenotypic traits. Under the assumption that evolution occurs from rural to urban populations, total observed trait change ($\Delta\bar{y}$, dashed line) can be partitioned into three components, as in Govaert et al. (2016): evolution (genotypic differentiation between rural and urban *Daphnia*), plasticity (here thermal plasticity given by the difference in trait values between 20 °C and 24 °C), and evolution of plasticity (difference in reaction norm slopes of rural and urban *Daphnia*). To visualize multivariate reaction norms in 3D-space, we first perform a Principal Component Analysis on the set of *n* phenotypic traits. The position of the group averages is then plotted for urban and rural populations at 20 and 24 °C along the first three PC axes; given that the amount of variation explained by the three PC axes rises, the Euclidian length of $\Delta\overline{x}$ represents the best approximation of the true length of $\Delta\bar{y}$.

The relative contribution of univariate and multivariate evolution, phenotypic plasticity, and the evolution of plasticity to total trait change

We quantified the relative contribution of both multivariate and univariate plastic (phenotypic plasticity to warming) and genetic (evolution of mean trait values and evolution of plasticity) changes to the total phenotypic trait change between rural and urban populations of *D. magna* under the prevailing temperature conditions in the field in the respective habitats (i.e. for the studied temperature range 20-24 °C). We applied the reaction norm approach described in Govaert et al. (2016) and Stoks et al. (2016) (see Fig. 2c,d). To this end, we assume that the rural populations are the 'ancestral' state (note they are characterized by a min. of 20% Biologically Valuable Area and thus reflect pristine Flemish habitats most closely) and that trait change between populations occurred during the transition of populations inhabiting colder, rural habitats to populations inhabiting warmer, urban habitats. We assume an average summer temperature of rural ponds of 20 °C and an average temperature increase in urban ponds of 4 °C, which broadly corresponds to the average summer temperature in rural ponds and the urban heat island effect measured in urban ponds (Brans et al. in press: difference in daily mean temperature between urban and rural ponds: ΔT_{mean} : +3.04 °C; difference in

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daily maximum temperature ΔT_{max}: +3.69 °C; data on a more limited set of ponds reported by Brans et al. 2017a indicate ΔT_{mean} : +3.93 °C and T_{max}: +4.03 °C; see also Fig. S1).

We additionally estimated 'evolutionary gain' for every trait, which quantifies the observed evolution-driven trait change achieved by urban compared to rural populations when exposed to warming. Evolutionary gain was calculated as the difference between the urban and rural mean trait values for each trait when reared at 24 °C, divided by the trait value of rural animals at 24 °C, and thus reflects the percentage of trait change that was additionally achieved through evolution when exposed to the warmer environment.

RESULTS

Multivariate MANOVA and univariate linear mixed-effect models

MANOVA results indicated overall significant differences in life history traits between rural and urban genotypes ($p < 0.001$; Table 1) and a significant plasticity response in these traits in response to rearing temperature ($p = 0.046$; Table 1). The associated univariate linear mixed-effect models showed that urban animals mature at a smaller size ($p = 0.003$, Fig. 3a) and earlier ($p = 0.019$, Fig. 3b), were younger at early reproduction (release first clutch: $p = 0.043$, release second clutch: marginally significant, $p = 0.050$) and had a higher fecundity (number of juveniles in second clutch: $p = 0.040$; first clutch: not significant, $p = 0.069$) (Fig. 3 c-f). Somatic growth did not significantly differ between urban and rural genotypes ($p = 0.386$, Fig. 3g), whereas performance (intrinsic population growth rate 'r') tended to be higher in the urban genotypes (marginally significant, $p = 0.051$, Fig. 3h). While for size at maturity a significant interaction effect (urbanization x temperature treatment) was found ($p = 0.036$, Table 1, Fig. 2a), MANOVA results indicate no overall effect of urbanization x

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temperature treatment on the combined set of response variables, thus indicating multivariate plasticity responses to temperature are similar for the urban and rural genotype sets. Phenotypic plasticity responses in response to increased rearing temperature for all traits were in the same direction as responses to urbanization: smaller size at maturity, earlier age at maturity and at release of clutches, higher number of eggs in the first clutch, higher somatic growth, and higher individual performance ('r') (Table 1, Fig. 3). Only number of juveniles in the second clutch did not significantly differ between animals reared at 20 and at 24 °C ($p = 0.123$; Table 1).

Model comparison of linear mixed-effect models with and without the random effect of clone within the rural and urban clone sets indicated significant effects of this factor for three out of eight variables (Table 1): individual performance ($p = 0.01$), age at maturity ($p = 0.006$), and size at maturity ($p < 0.001$). This indicates significant clonal variation [for these three traits] within the urban and rural clone sets for these traits.

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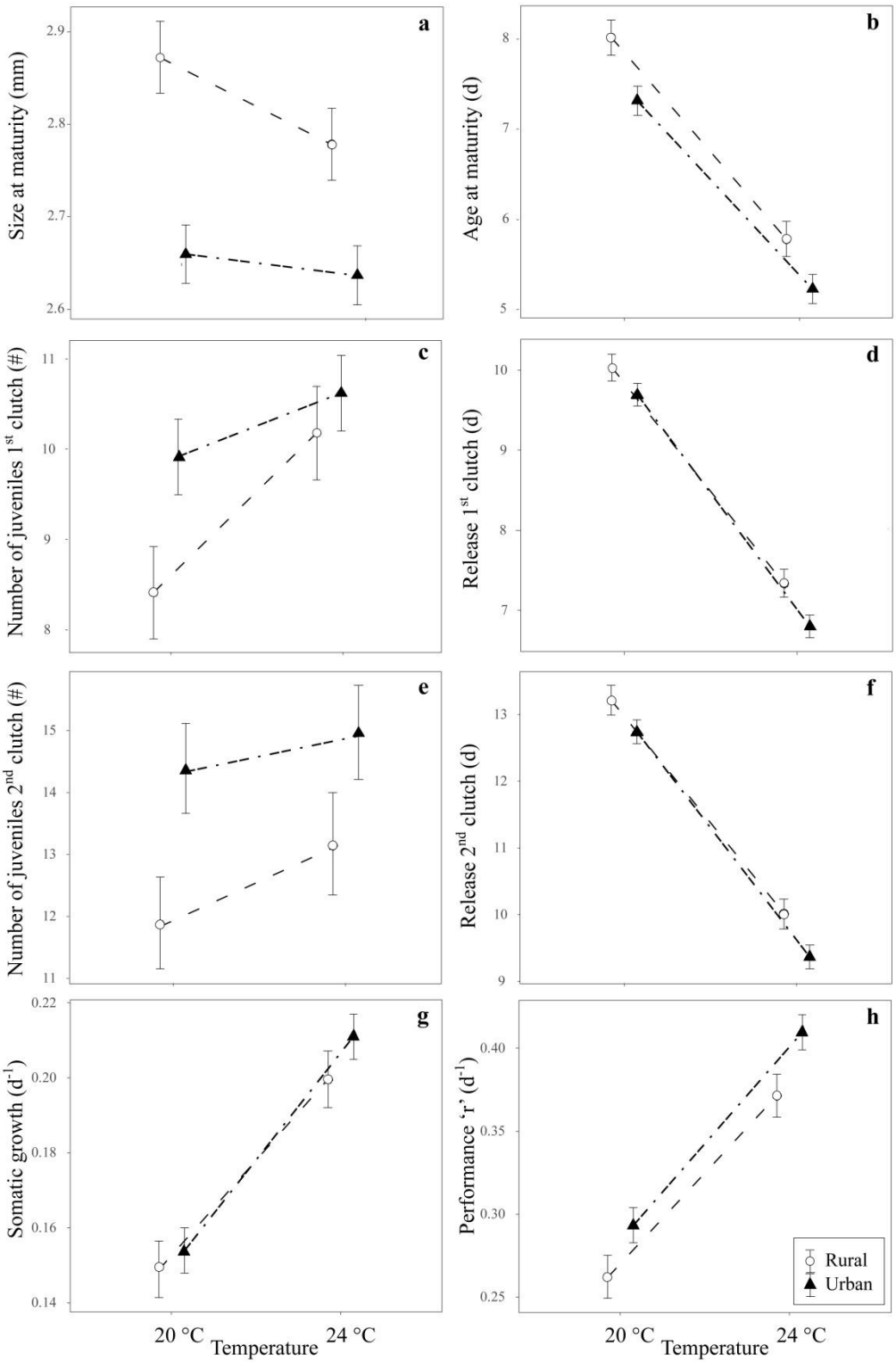

Figure 3. Univariate results. Mean (± 1 SE) size at maturity (a), age at maturity (b), number of juveniles in and the timing of release of the first (c-d) and second (e-f) clutch, somatic growth (g), and individual performance (h), for urban (black triangles) and rural (white circles) *Daphnia*, as a function of rearing temperature (20 °C, 24 °C; reaction norms: dashed lines; n = 220 per temperature treatment). Means and standard errors are obtained via model outputs of the linear mixed-effect models, accounting for random and nested data structure.

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Multivariate trait space and trajectory analysis

PTA and convergence/divergence analysis reveals no significant difference in the size and angle of the multivariate phenotypic plasticity trajectories between urban and rural *D. magna* genotype sets (with both genotype sets being represented by multiple populations and genotypes nested in populations) when reared at 24 °C versus 20 °C (Fig. 4a,b, dashed lines, size difference = 0.304: p = 0.179; angle θ = 8.970°: p = 0.138). There is also no significant difference in size and angle of the evolutionary trajectories comparing rural and urban clone sets at each temperature (Fig. 4a,b, full lines, size difference = 0.031, p = 0.845; angle θ = 26°, p = 0.746). However, we find a clear difference in position in multivariate trait space between urban and rural populations (i.e. Euclidian distance between urban and rural centroids significantly different from 0; $p < 0.001$ at both temperatures), confirming earlier MANOVA results. The first three PC axes cumulatively explain 81.6% of multivariate trait variation and are strongly correlated with development time (age at maturity, at release of first, and at release of second clutch), somatic growth and performance (PC1, 49.2%), fecundity (PC2, 20.7%), and size at maturity (PC3, 11.7%). Combining the results of PTA and PCA indicates that both phenotypic plasticity in response to rearing at higher temperature (in rural as well as in urban populations) and genotypic differentiation (evolutionary trajectories) between rural and urban populations results in a shift towards a faster life style, with shorter development time, higher potential intrinsic rate of population growth, higher fecundity, and smaller body size.

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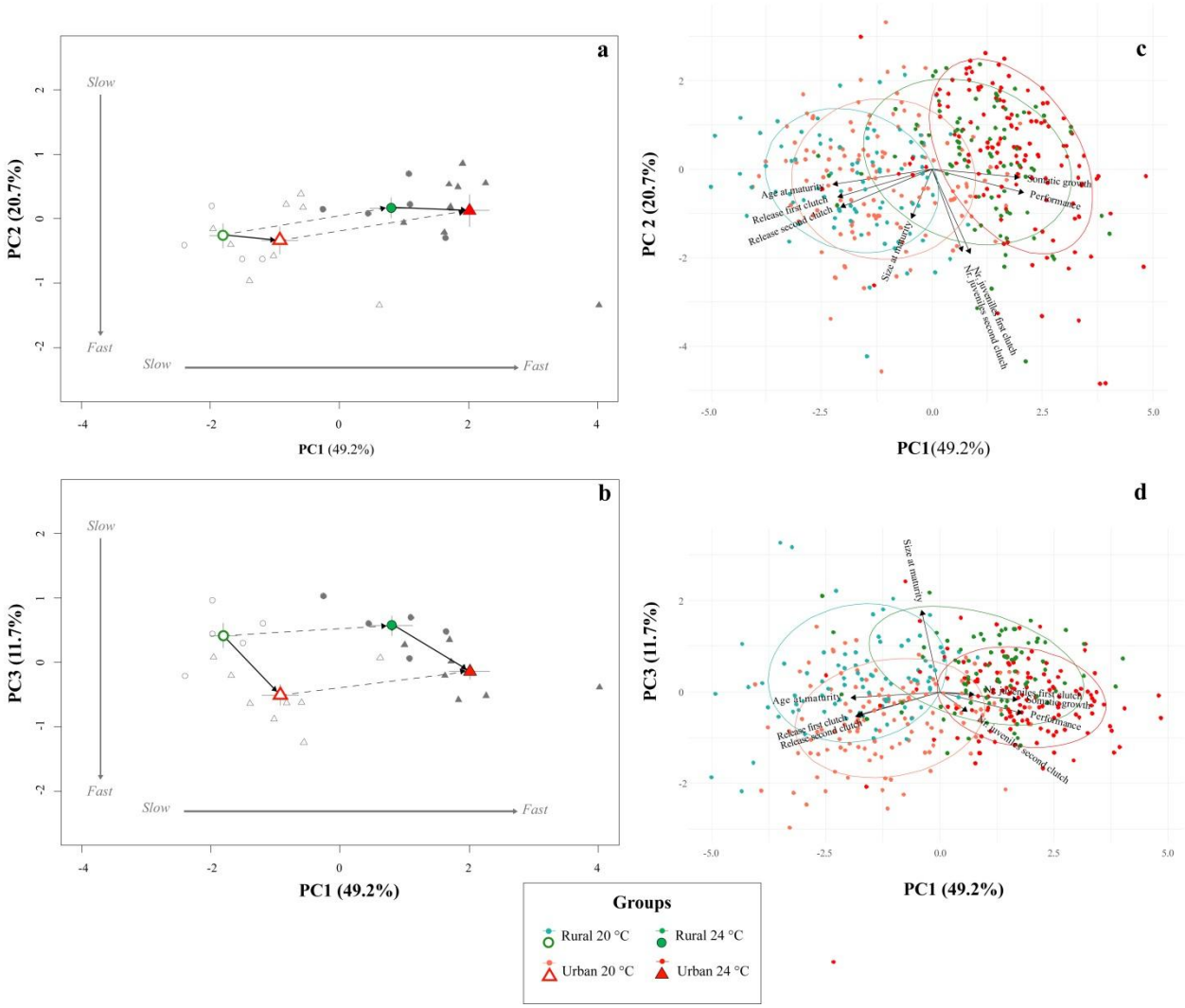

Figure 4. Multivariate results. Principal Component Analysis plots showing: (a,b) phenotypic plasticity trajectories (multivariate response to experimental warming for eight morphological and life history traits; dashed lines) and evolutionary trajectories (multivariate genetic differentiation between rural and urban animals; full lines) for rural (green circles) and urban (red triangles) populations of the water flea *Daphnia magna* reared at 20 (open symbols) and 24 °C (full symbols); population means are depicted in grey shades (white: 20 °C, gray: 24 °C), average 'urban' and 'rural' centroids are depicted in the according color (rural: green; urban: red); (c,d) PCA plots showing all observations (n = 440) grouped according to the urbanization x temperature combinations (rural-20 °C: blue, rural-24 °C: green, urban-20 °C: orange, urban-24 °C, red). Patterns are given for PC1 and PC2 (a,c) and PC1 and PC3 (b,d). An indicative 'pace-of-life' axis along all PC axes is given in plots a and b.

Contributions of evolution, plasticity, and the evolution of plasticity to total trait change

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We found a high relative contribution of evolution (i.e. evolution of a mean trait value combined with evolution of plasticity) to total observed trait change between rural and urban populations, assuming a shift from 20 to 24 °C, for traits: size at maturity (75.11%), number of juveniles in the first (59.18%) and number of juveniles in the second clutch (71.21%; Fig. 5a,b). Although lower, the relative contribution of evolution still accounts for ca. 25% of total trait change in two traits linked to pace-of-life: age at maturity (27.60%) and individual performance (25.96%). Within the component of evolutionary change, evolution of mean trait value contributes for all traits more to overall trait change (range 10.47% - 56.30%) than evolution of phenotypic plasticity, except for somatic growth (8.05% compared to 10.40%). Relative contributions of plasticity to the total observed phenotypic trait change (i.e. from rural populations reared at 20 °C to urban populations reared at 24 °C) is highest for release of first and second clutch (for both traits 83.25%) and somatic growth (81.57%). For multivariate trait change, evolution contributes 28.16% to total observed change in multivariate trait space (Fig. 5b). Evolutionary gain (Fig. 5c; Table S2, Supplementary Information B) is highest for performance (an increase of 10.30%), age at maturity (a reduction in time of 9.56%), and release of the first clutch (a reduction in time of 7.37%).

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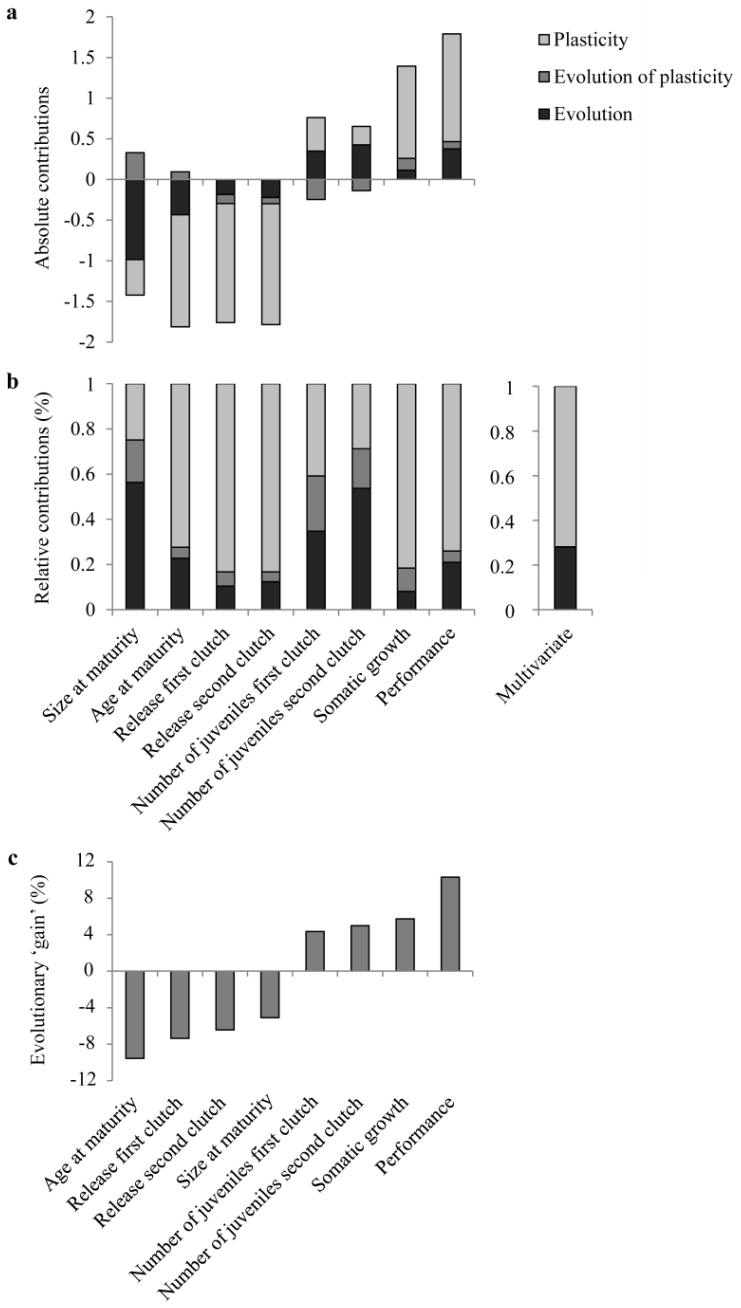

Figure 5. Eco-evolutionary variation partitioning. (a) Absolute and (b) relative contributions of evolution (black), evolution of (thermal) plasticity (dark grey), and phenotypic plasticity (light grey) to the standardized phenotypic change measured in eight life hisotory traits in urban and rural populations of *Daphnia magna*, reared at 20 and 24 °C (total nr. of observations: n = 440). Contributions are calculated according to Stoks et al. (2016) using the additive method. (c). Evolutionary gain, calculated for eight life history traits in urban and rural *Daphnia magna* populations. Evolutionary gain is given as the percentage and direction of trait change for each trait (non-standardized, unit specific measure) that is realized due to evolution at the high temperature (i.e. comparing trait values of rural populations at 24 °C with trait values of urban populations at 24 °C). Evolutionary gain is ranked according to direction and percentage in the plot.

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Table 1. Statistical results. Results of the MANOVA and univariate linear mixed-effect models, testing for the effect of urbanization level, treatment, and their interaction on eight morphological and life history traits measured in *Daphnia magna*. Significant results ($p < 0.05$) are given in bold . Marginally significant results ($p = 0.05$) are marked with a '•'.

	Urbanization				**Temperature**				**Urbanization x Temperature**				**Clone**	
MANOVA	**F**	**Df**	**Pillai**	**P**	**F**	**Df**	**Pillai**	**P**	**F**	**Df**	**Pillai**	**P**		
All traits	5.119	8	**0.455**	**< 0.001**	2.171	8	0.262	**0.046**	1.453	8	0.192	0.199	*Included as error term*	
Univariate linear models	**F**	**Df**	**Df_{res}**	**P**	**F**	**Df**	**Df_{res}**	**P**	**F**	**Df**	**Df_{res}**	**P**	**χ^2**	**P**
Size at maturity	13.893	1	11.153	**0.003**	9.495	1	361.096	**0.002**	4.451	1	361.152	**0.036**	5.376	**< 0.001**
Age at maturity	7.525	1	10.845	**0.019**	403.273	1	364.024	**< 0.001**	0.468	1	364.183	0.494	7.534	**0.006**
Release 1st clutch	5.241	1	10.656	**0.043**	664.724	1	366.870	**< 0.001**	0.819	1	366.973	0.366	0.697	0.404
Number of juveniles 1st clutch	3.443	1	10.820	0.069	7.845	1	364.920	**0.005**	1.674	1	365.060	0.197	0.106	0.745
Release 2nd clutch	4.843	1	10.690	**0.050•**	655.013	1	366.61	**< 0.001**	0.402	1	366.730	0.526	0.004	0.998
Number of juveniles 2nd clutch	5.446	1	10.592	**0.040**	2.384	1	362.141	0.123	0.537	1	362.345	0.464	3.005	0.083
Somatic growth	0.816	1	10.840	0.386	281.354	1	366.162	**< 0.001**	0.932	1	366.316	0.335	0.272	0.602
Performance	4.777	1	11.139	**0.051•**	476.707	1	362.030	**< 0.001**	0.463	1	362.120	0.496	6.655	**0.010**

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DISCUSSION

Cities generate strong and fast abiotic and biotic environmental disturbance gradients (Seto et al., 2012; Parris, 2016). So far, urban evolutionary studies on aquatic ectotherms are extremely limited (Brans et al., 2017a,b; Tüzün et al., 2017). Due to the urban heat island effect, water temperatures in small, standing waterbodies such as ponds and shallow lakes are considerably higher in urban than rural settings ($\Delta T_{mean\text{-}summer}$ = 3.04 °C; $\Delta T_{max\text{-}summer}$ = 3.69 °C; Brans et al., in press, Brans et al., 2017a), and this likely induces both thermal stress and oxygen limitation (Pörtner, 2001). Our results show that urban populations of the water flea *Daphnia magna* evolved a 'fast' life history strategy and that both rural and urban populations exhibit a strong, nearly identical, plastic response to the applied change in rearing temperature (20 °C versus 24 °C) for all measured traits. The direction of change, towards a faster pace-of-life, is very similar for the plasticity responses when exposed to higher temperature and for the genetic trait shift as one moves from rural to urban populations, for each of the individual life history traits as well as for multivariate trait trajectories. Given the strong, intrinsic association between temperature and life history traits in ectotherms, our results suggest that anthropogenic warming in cities shapes evolutionary pace-of-life strategies. Other stressors linked to urban environments, such as increased exposure to disturbances (such as heat waves and artificial management; Hassall, 2014; Wouters et al., 2017; Brans et al., in press) might also have contributed to the observed genetic differentiation in pace-of-life in our study system. We discuss our results in more detail in the next paragraphs.

We found pronounced genetic differentiation in multiple life history traits when considered individually (Table 1, Fig. 2) as well as in a multi-trait perspective (MANOVA, evolutionary trait trajectories Fig. 3 and Fig. 4). These genetic changes were along the slow-to-fast axis, with urban *Daphnia* populations being shifted towards the fast end of the 'pace-

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of-life' continuum. Urbanization causes a genetic shift towards faster maturation and faster release of early clutches, higher early fecundity, higher potential population growth rate, and smaller size at maturity. This response is, given the observed anthropogenic warming in the region (De Ridder et al., 2015; Wouters et al., 2017; Brans et al., in press), in agreement with expectations of life history adaptation to warming (cf. temperature-size rule; Atkinson, 1994; Angilletta, 2009). While the small body sizes of urban genotypes might be due to selection for short developmental time, it is most likely also due to or reinforced by the fact that smaller animals have a higher thermal tolerance (Geerts et al., 2015; Brans et al., 2017b). Although rapid maturation and small body sizes often come at a fecundity cost (Clarke, 2003), urban genotypes had a higher fecundity. This suggests selection for higher population growth rates (intrinsic population growth rate *r*). The observed genetic trait changes as one moves from rural to urban populations are indeed similar to those expected under "r"-selection (r-K selection; MacArthur & Wilson, 1967; Pianka, 1972; Stearns, 1976; Reznick et al., 2002). While our observation of a shift in pace-of-life shift linked to urbanization is in general correspondence with expectations under a scenario of adaptive evolution in response to the urban heat island effect, we do not preclude that other stressors linked to urbanization may equally have impacted this shift. A survey of local environmental conditions in a set of 83 ponds (Jessie Engelen, Caroline Souffreau and others, unpublished data; see also Gianuca et al 2018) did not reveal strong and systematic differences in nutrient concentrations (eutrophication), salt pollution, pesticide load and metal contamination along the urbanization gradient. Yet, our observation of a change in pace-of-life might reflect adaptation to a higher incidence of mechanical and hydrological disturbances (e.g. pond management: clearance of the litter layer, addition and removal of water) or extreme temperatures that are likely more prominent in urban compared to rural ponds (Wouters et al., 2017; Brans et al., in press; KIB personal observations).

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Our study involved multiple genotypes isolated from a total of 13 populations along independent urbanization gradients in Flanders (Northern Belgium). The observation that the differentiation among rural and urban populations was highly repeatable, as illustrated by the population averages plotted in Fig. 3, indicates that the observed evolutionary changes are a general response to urbanization in this species in the study region (see also Johnson et al., 2015; Thompson et al., 2016; Tüzün et al., 2017). Our results on local adaptation at short spatial scales (Richardson et al., 2014) are in line with previous studies providing evidence for adaptive genetic trait change to other anthropogenic disturbances in water fleas over short time scales (months to a few years) and spatial distances (Hairston et al., 2005; Geerts et al., 2015; Jansen et al., 2015; Zhang et al., 2016), and with our earlier results on the evolution of a higher thermal tolerance in city populations (Brans et al., 2017b). We obtained evidence for clonal variation in genotypic trait values within populations for three out of eight measured traits in both urban and rural populations, indicating that both sets of populations harbor the capacity to respond in their life histories to further temperature increases either due to enhanced urbanization or to climate warming.

The common garden approach we used for testing genotypes at two temperatures, approximately corresponding to the temperature difference between urban and rural habitats (Brans et al., in press, see also Fig. S1), allowed assessing relative contributions of evolution phenotypic plasticity, and evolution of plasticity to total trait change (Merilä & Hendry, 2014; Govaert et al., 2016; Stoks et al., 2016; Diamond & Martin, 2017) under an urbanization scenario. This scenario assumes that the urban heat island effect results in a temperature increase of approximately 4 °C, and that average summer temperatures in rural systems are around 20 °C (Brans et al., 2017a; Brans et al., in press; De Ridder et al., 2015). Evolution accounted for a substantial portion (30%) of total observed multivariate phenotypic trait change (i.e. from rural populations at 20 °C to urban populations at 24 °C). Evolutionary

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divergence of city compared to rural populations was more important than phenotypic plasticity to a temperature difference of 4 °C (corresponding to the average temperature difference between city and rural ponds) of the ancestral population for size at maturity and number of juveniles in the first and second clutch, while plasticity in response to the 4 °C difference in rearing temperature accounted for the majority of trait change observed in traits related to developmental rate, performance, and somatic growth. Plasticity is an important first crucial buffer against the impact of (anthropogenic) environmental change and habitat disturbance (Gienapp et al., 2008; Donnelly et al., 2012; Merilä & Hendry, 2014). It is becoming increasingly clear, however, that acclimatization alone might not guarantee long term population persistence under strong anthropogenic pressure (Visser, 2008; Franks et al., 2014; Duputié et al., 2015; Gunderson & Stillman, 2015). While phenotypic plasticity can feed evolutionary processes via genetic assimilation (Waddington, 1953; Lande, 2009; Ehrenreich & Pfennig, 2016), our study provides no direct evidence for a process that would turn phenotypic plasticity into genetic trait differentiation, given that overall, urban populations show the same univariate and multivariate plasticity responses to increased temperatures as rural populations, while they have strongly diverged in mean trait values.

Phenotypic plasticity responses to temperature increase were remarkably similar in urban and rural genotypes, except for size at maturity, for which we observed reduced plasticity in the city populations (see also Brans et al., 2017a). Although this absence of evolution of plasticity contrasts to results on other systems and what is expected under anthropogenic disturbance scenarios (Reed et al., 2011; Chevin & Hoffmann, 2017; Diamond & Martin, 2017), it might indicate there are constraints involved. For instance, we observe the strongest parallel responses for traits that are linked to rates (age at maturity and release of first and second clutch, somatic growth, performance). This may reflect the intrinsic relationship between metabolic rates and temperature (Brown et al., 2004). The only trait for

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which we observe an urbanization x temperature interaction is size at maturity (urban animals here show reduced size-plasticity upon experimental warming compared to rural animals), which is also the trait for which we observe the strongest contribution of evolution. We hypothesize that this is due to the added selection for small body size imposed by the need to increase thermal tolerance (Geerts et al., 2015; Brans et al., 2017b), and that the reduced plasticity in urban animals is due to a trade-off associated with costs of very small body size at maturation.

As an increasing number of studies report genetic adaptation to urbanization in organisms ranging from plants (Cheptou et al., 2008; Johnson et al., 2015; Thompson et al., 2016) over insects (Diamond et al., 2017; Tüzün et al., 2017) to birds (Atwell et al., 2012; Mueller et al., 2013), understanding and predicting how populations and species respond to regional and local increases in urbanization will require insights on evolutionary trait change. For all eight studied traits, trait changes that result from adaptation to urbanized settings are in the same direction as the plasticity-mediated responses to temperature increase (Fig. 4a). While the evolution of plasticity is in the opposite direction compared to evolution of mean trait value for four out of the eight traits (Fig. 4a), this component was small for all traits, and the evolution of mean trait value is for all traits in the same direction as the plasticity responses. This is in line with the idea that the urban heat island effect might be one of the important drivers of the observed evolutionary pace-of-life shift in urban *Daphnia*. We thus have no evidence for thermal countergradient adaptation for life history traits in this system (as was recently observed by Tüzün et al., 2017, for damselflies in the same region). As a result, the net change in trait value due to combined plasticity and evolution is large (Table S2, Supplementary Information B). For example, size at maturity changes from 2.87 mm to 2.63 mm (an 8.2% reduction), whereas age at maturity changes from 8.0 days to 5.2 days (a 34.8% reduction), and performance from 0.26 day^{-1} to 0.41 day^{-1} (a 56.2% increase). Given

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the additive nature of the net contribution of evolution (i.e. evolution of mean trait and of trait plasticity combined), it is also straightforward to calculate the additional "gain" in a trait value that is achieved in urban populations in the warmer environment (24°C) as a result of evolution. This gain is considerable, being approximately 10% for age at maturity and performance (as compared to the trait value in the absence of evolution). This may have important consequences of the functioning of urban ecosystems and species interactions. In this context, it is important to note that evolutionary trait changes might differ widely among organism groups in amplitude and direction. For instance, while Tüzün et al. (2017) observed a countergradient response to the urban heat island effect in damselfly larvae, we here report a genetic response that is enhanced by the environmental effect of urban warming. As a result, evolutionary responses may either dampen or increase the degree of trophic mismatches along urbanization gradients, possibly impacting food web structure and dynamics (Clarke, 2003; Durant et al., 2007; Visser, 2008; De Block et al., 2013; Bonaviri et al., 2017; Start et al., 2017. The consequences of such evolution-driven mismatches have, however, not yet been quantified in the context of urbanization.

Strikingly, the genetic trait changes associated with adaptation to urban heat island and hot-tub effects that we report here (life history traits) and in earlier work (Brans et al., 2017b; thermal tolerance) broadly match observed genetic changes in relation to climate warming in *Daphnia* (Geerts et al., 2015) as well as observed latitudinal variation in life history traits (Sarma et al., 2005) and thermal tolerance (Yampolsky et al., 2014; Geerts et al., 2015) in invertebrate ectotherms. Urbanization-induced genetic life history changes show striking parallels with climate change-driven trait change during the past 60 years, with animals becoming smaller, maturing earlier, reproducing faster, and showing increased voltinism (Parmesan & Yohe, 2003; Daufresne et al., 2009; Altermatt, 2010; Bestion et al., 2015). This suggests that urban populations might be partially pre-adapted to climate change,

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which can profoundly impact spatio-temporal dynamics of species' responses to future climate change (Van Doorslaer et al., 2009; Urban et al. 2012, 2016).

We conclude that urbanization strongly shapes pace-of-life evolution in *Daphnia magna*. Urban *Daphnia* mature earlier, have a smaller size at maturity, reproduce faster, produce larger clutches, and have a higher potential population growth rate when compared with rural populations. Univariate and multivariate thermal reaction norms show strong responsiveness to warming, and are similar in urban and rural populations. Plasticity responses upon warming and evolutionary trait shifts with increasing urbanization are in the same direction, which results in additive effects and pronounced trait changes as one moves from rural population at rural temperatures (approximately 20 °C in the study region) to urban populations at temperatures simulating the urban heat island effect (here: 24 °C). The implications of the observed evolutionary responses can be manifold. For example, if in the absence of evolution these populations would not have been able to thrive in the urban environment (cf. evolutionary rescue; Gomulkiewicz & Holt, 1995), this would likely have a strong impact on the functioning of urban ponds, as large-bodied *Daphnia* are among the most efficient grazers on phytoplankton (Brooks & Dodson, 1965; Mourelatos & Lacroix, 1990; Gianuca et al., 2016). The evolutionary shift to a faster pace-of-life and smaller body sizes may, however, also by itself lead to a reduced capacity of the *Daphnia* populations to top-down control algae (Goitom et al., 2017). Finally, in the context of climate change, our results suggest that urban populations, in addition to already being adapted to higher temperatures, still harbor genetic variation that offers evolutionary potential for further adaptation to increased temperatures. In a more regional and subcontinental setting, it is possible that genetic adaptation to higher temperatures in urban areas might give urban populations an advantage over rural ones in the context of climate change, potentially resulting in urban populations re-invading rural settings. While the likelihood of such a

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scenario depends amongst others on the degree to which differences in urban and rural conditions, apart from temperature, would be more influential than pre-adaptation to higher temperatures, it is worth exploring its likelihood and potential consequences.

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Author contributions and competing interests

KIB and LDM developed the general design of the study, the experiments and analyses. KIB performed the experiment, carried out the statistical analyses with input from LDM, and wrote the first version of the manuscript. LDM edited the first version, and KIB and LDM contributed equally to the further editing of subsequent versions of the manuscript. The authors declare no competing interests.

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Data accessibility

Data are available online (Brans & De Meester, 2018; Data from: City life on fast lanes: urbanization induces an evolutionary shift towards a faster life style in the water flea *Daphnia*. Dryad Digital Repository, DOI: doi:10.5061/dryad.hm63867).

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