Supplementary Information B "Urbanization drives genetic differentiation in physiology and structures the evolution of pace-of-life syndromes in the water flea *Daphnia magna*"

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B. MATERIAL AND METHODS

Quantification of response variables

Energy storage compounds

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

After a 5 minute incubation (25 °C) with the Bradford Reagens (Sigma-Aldrich B6916, [1]), total protein content was measured (at 595 nm) in quadruplicate and the means per pooled sample were used for the statistical analyses. Total protein concentration was expressed in µg per µg dry mass of the set of animals that were homogenized.

To quantity total fat content we followed the protocol of Bligh & Dyer ([2]). H_2SO_4 (100%) was added to the homogenate, which was left to incubate at 150 °C (20 min.). After cooling down, total fat content was assessed in technical triplicate by measuring absorbance at 340 nm. Fat concentration was calculated based on a standard curve of glyceryl tripalmitate and expressed as µg per µg dry mass.

Total sugar content (glucose + glycogen) was measured following the protocol described in Stoks et al. ([3]) using a glucose oxidase kit (MAK097-1KT, Sigma Aldrich). We first added an

amyloglucosidase solution (Sigma A7420) to the homogenate, which was then left to incubate at 37 °C for (30 min.). In a next step a glucose reagent was added to the same sample and left to incubate at 30 °C for 20 min. Absorbance was measured at 340 nm. To distinguish glycogen from glucose components, the second step was repeated on a new subsample of the homogenate, after which the resulting concentration was subtracted from the first measurement (i.e. (glucose + glycogen) – glucose). Total sugars (glucose + glycogen) was used as response variable in the statistical analysis.

Antioxidant defense

The activity of superoxide dismutase (SOD), catalase (CAT), two known key antioxidant enzymes in insects and invertebrates [4], and gluthation-S-transferase (GST), a secondary anti-oxidative enzyme which protects against oxidizing and toxic substances by detoxifying ROS-damaged cellular components was measured as an estimate of antioxidant defense.

SOD activity was quantified using the protocol of De Block & Stoks ([5]), based on the SOD assay kit WST (Fluka, Buchs, Austria). WST working solution was mixed with enzyme working solution and the homogenate of the sample. After incubation (20 min. at 37 °C), absorbance was measured at 450 nm. SOD acitivity is defined as the percentage inhibition of the reduction reaction of WST-1 with superoxide anion and is standardized per µg protein.

CAT activity was measured following De Block & Stoks ([5]); after a homogenate dilution with PBS (16 times), 100 µl of 20 mM H_2O_2 was added (to 20 µl of the diluted sample and 80 µl PBS (100 mM, pH 7.4)). CAT activity was then quantified as the degradation rate of H_2O_2 with absorbance being measured (duplicate measurements) every 30 seconds (12 readings) at 240 nm.

Using the slope of the linear part of the reaction curve, CAT activity was calculated as the amount of H_2O_2 decomposition per minute and per µg protein.

GST activity was quantified based on the protocol of McLoughlin et al. ([6]). 5 µl of sample was added to 195 µl of substrate solution (2 mM glutathione (GSH) solution, and 1 mM 1-chloro-2,4-dinitrobenzene (CDNB) solution). The filled 96 well microtiter plate (suited for UV-spectrum) was pre-incubated for three minutes at 30 °C, after which absorbance was read in duplicate at 340 nm every 30 seconds for 10 min at 30 °C. GST activity was calculated based on the slope of the linear part of the reaction plot, as the amount of GS-DNB formed per minute and per µg protein.

Oxidative damage

A HPLC-based TBARS essay was conducted to measure malondialdehyde (MDA) levels [7], a byproduct of lipid peroxidation. Sample preparation was done as in Debecker et al. ([8]): 50 µl of homogenate sample was mixed with an equal amount of thiobarbituric acid (TBA; 0.4% (40 mg TBA in 10 ml 0.2 M HCl)). After a 1-hour incubation at 90 °C, 165 µl butanol (100%) was added to the sample, which was mixed and centrifuged (3 min., 4 °C, 13000 rpm). 50 µl of this supernatant was then injected in an HPLC/UV-Vis system with a 10 µl loop on a C18 column (250 x 4.6 x 5 µm; chromatogram measurements at 535 nm). MDA concentrations were expressed as nmol MDA per µg dry mass. Total fat content was used as a covariate in the statistical analysis.

Statistical analysis

MANOVA and linear mixed-effect models

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 on all response variables scaled to a mean of 0

and standard deviation of 1. Fixed factors were urbanization, temperature treatment, and their interaction term. A random error term for 'Clone' (i.e. genotypic identity of each line; see Table S1 of Supplementary Information A) nested in population was added. Pillai's trace test statistic was extracted using the 'car' package [9]. Next, we ran separate linear mixed-effect models ('lme4' package, [9]) for each of the seven response traits (same model structure; fixed effects: urbanization level, temperature treatment, and urbanization x temperature treatment; random effects: clone, nested in population, and population, nested in urbanization class). F-test statistics were computed using the 'car' package [9]. F-tests, were fitted with the restricted maximum likelihood ratio; degrees of freedom were corrected using the Kenward-Roger approximation. To assess a possible effect of clonal identity (implying genetic variation for a certain trait within populations and thus evolutionary potential), we refitted each linear mixed-effect models without this random factor and performed a model comparison (likelihood ratio test with χ^2 distribution; p-values were halved according to 1-tailed testing).

Assumptions of normality and homogeneity of variances were checked for each model visually (by plotting model residual histograms, QQ-plots, and residual vs. fitted values) and tested for by performing Shapiro-Wilk's test of normality on model residuals and Levene's test for homogeneity of variance on each variable (using either treatment or urbanization as a grouping factor). All model assumptions were met except for MDA, which was log-transformed to better meet residual normality.

Structural equation modelling

First we assessed trait covariation patterns in life history traits and physiology traits separately. In a last SEM analysis we assessed the possible presence of an overarching POLS by integrating both

life history and physiological traits. For each SEM [10], we compared four alternative models with different combinations of factor loadings (i.e. path coefficients) constrained to be equal vs. freely varying across all urbanization x temperature groups (i.e. urban-20 °C, rural-20 °C, urban-24 °C, rural-24 °C; Table S2, Supplementary Information A): (I) loadings were constrained to be equal across temperature treatment and urbanization level, (II) loadings were constrained to be equal across temperature treatments only, (III) loadings were constrained to be equal across urbanization level only, and (IV) loadings are all free (Table S2). For example, if a model with loadings constraint across temperature treatments (model II) is regarded the most appropriate model explaining the trait covariation patterns, it in essence indicates trait covariation patterns are the same in animals reared at 20 or 24 °C, but are different for urban vs. rural populations. This thus means urbanization (i.e. evolutionary processes) shapes trait covariation patterns, given that rural and urban populations are characterized by different factor loadings and consequently different trait covariation patterns. If a model with loadings constraint across urbanization level is the most appropriate (model III), it indicates trait covariation patterns are similar between urban and rural populations, but different when comparing animals reared at 20 °C vs. 24 °C. In case model selection leads to model IV being the best fit, indicated by factor loadings freely varying across all urbanization x temperature treatment groups, trait covariation patterns differ between urban animals reared at 20 °C, urban animals reared at 24 °C, rural animals reared at 20 °C, and rural animals reared at 24 °C. In the last case, both urban background (evolution/genetic differentiation) and temperature treatment (plasticity to warming), impact trait covariation patterns.

Based on AIC scores the most appropriate model was chosen. In case $\Delta AIC < 2$, we assessed model weights (*W*) and evidence ratios (E.R.) [11,12] to validate model choice. *W* characterizes the probability the evaluated model is the most appropriate, given the set of models

tested. E.R. is calculated by dividing the model weight of the best model (lowest AIC) by the weight of the evaluated model and thus represents the likelihood of the best model compared to the evaluated model.

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 [13]. Path coefficients of two contrasted groups were subtracted from each other and divided by the pooled standard error, after which values were evaluated as t-values and significances were assessed. P values were corrected for multiple testing using Bonferroni corrections (paths significantly differing - $p < 0.05$ - between groups of interest will be indicated with a †).

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