Title

Trehalose provisioning in *Daphnia* resting stages reflects local adaptation to the harshness of diapause conditions

Authors

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Abstract

Environmental fluctuations often select for adaptations such as diapause states, allowing species to outlive harsh conditions. The natural sugar trehalose which provides both cryo- and desiccation-protection, has been found in diapause stages of diverse taxa. Here, we hypothesize that trehalose deposition in resting stages is a locally adapted trait, with higher concentrations produced in harsher habitats. We used resting stages, produced under standardised conditions, by 37 genotypes of *Daphnia magna* collected from Western Palearctic habitats varying in their propensity to dry in summer and freeze in winter. Resting eggs produced by *D. magna* from populations from summer-dry habitats showed significantly higher trehalose than those from summer-wet habitats, suggesting that trehalose has a protective function during desiccation. In contrast, winter-freezing did not explain variation in trehalose content. Adaptations to droughts are important, as summer dryness of water bodies is foreseen to increase with ongoing climate change.

Keywords

Trehalose; desiccation; diapause; local adaptation, Daphnia magna

Introduction

Environmental factors determine the occurrence and geographic range of species, whose ability to persist in any given habitat depends on their capacity to develop behavioural, physiological or structural adaptations, especially to extreme environmental fluctuations (1,2). These adaptive strategies, which have allowed most environments on the planet to be colonized (3), may involve moving to a different place (migration (4)) or suspending development and forming protective dormant stages (5).

Diapause, a programmed state of developmental arrest, is a form of dormancy often initiated in response to environmental triggers in anticipation of deteriorating environmental fluctuations (6–8). It often goes hand-in-hand with seasonality (9). During diapause, activities such as embryogenesis, growth, maturation, breeding and hatching may be postponed, resulting in dormant cysts, gemmules, eggs and larvae (8–10), capable to survive conditions like cold or heat stress (5). Desiccation, a state of extreme dryness, is frequently observed as a stress factor that triggers diapause in many organisms. In diapause, some organisms can successfully survive desiccation, even when 99% of the water is removed from their cells (11,12). This ability seems to have evolved early in evolutionary history and is observed in prokaryotes (13) and eukaryotes, including plants (14), fungi (15) and animals (7). Specific mechanisms and adaptations, including the production of sugar molecules (e.g. sucrose and trehalose) (11,16,17) and small stress proteins (e.g. heat shock and late embryogenesis abundant proteins) (9,18,19) are required to survive the severe damage that desiccation would otherwise cause to cells (20). The array of these mechanisms suggests the convergent evolution of those traits (reviewed in (20)).

The role of the natural sugar, trehalose, was first identified as being essential to diapause in *Artemia salina* (21,22), whose dormant eggs contained higher trehalose concentration than non-dormant eggs (22). Trehalose not only helped organisms survive during diapause conditions, but also functioned as an energetic substrate, boosting their emergence from diapause and their further development (21). Numerous studies have corroborated the role of trehalose as both a cryo- and desiccation-protectant, for instance in bacteria (23), fungi (24–26), nematodes (27), tardigrades (28), insects (29,30) and crustaceans (31). Trehalose also aids organisms in other stressful conditions, such as when water salinity and temperature rise (19,20,32). The main benefits of trehalose are its stability as a chemical with a low degradation rate; it is able to stabilize dry membranes, liposomes and proteins over the long-term by impeding their aggregation (27,33) and has a special ability to reach a vitrification state and fill cellular spaces left by water (11,22,25,34). However, despite the fact that trehalose is a compatible solute in

many organisms (35), its biosynthesis is energetically costly (26). Furthermore, over-accumulations of trehalose have led to aberrations and seem to interfere with reactive oxygen species signalling and reducing programmed cell damage (36). Thus, trehalose is a double-edged sword that should only be relied on when its benefits outweigh its costs. If these benefits depend on the environment, trehalose expression should show a signature of local adaptation, occurring in higher amounts in habitats with more severe diapause conditions.

Here, we test whether the concentration of trehalose in resting eggs is higher in genotypes of *Daphnia magna* from habitats with particularly harsh diapause conditions, namely water bodies that freeze in winter and desiccate during summer. *Daphnia magna* Straus 1820 is an ideal organism to study local adaptation in diapause. It inhabits brackish and freshwater bodies in a wide variety of habitats, from permanent to intermittent freshwater ponds (37) and it produces diapausing resting eggs which ensure survival during severe, otherwise unliveable conditions (38,39).

Material and methods

Daphnia *samples*

Thirty-seven genotypes, each from a distinct population across the Western Palearctic were selected from the *Daphnia magna* Diversity Panel (e.g. (40); Figure 1, Table S1). Water bodies were characterized by their tendency to dry out during summer or not (based on observation and reports, see (40)), and to freeze regularly during winter or not (indicated as average temperature of the coldest month below zero). This resulted in four distinct habitat categories ((41,42); Figure 1, Table S1).

Resting stage production

Resting eggs were produced by selfing from genotypes kept as clonal lines for five months under standardized laboratory conditions. The number of resting eggs produced depends on genotype (42), but can be triggered by short photoperiod or crowding (38,43). We kept crowded monoclonal populations at 16 and 20 °C and 8:16 dark:light cycle in 400-mL medium jars with artificial medium (44), feeding three times per week with the green algae *Scenedesmus sp.*. Medium was changed once a month. Resting eggs were collected weekly and kept in closed jars with the same medium conditions for 5 months maximum.

Trehalose extraction and concentration measure

For each genotype, eggs (actually embryos in developmental arrest) were removed from the resting egg-case. In total eight biological replicates per genotype were used, each containing five eggs. For each replicate, we calculated the total egg volume (assuming the eggs were ellipsoids) by measuring length and width per egg using an eyepiece graticule (2mm ± 0.01) in a stereomicroscope. Eggs were cleaned with deionized water, placed in a 0.5-mL Eppendorf tube filled with 25 µL of ultrapure water and disintegrated using a sonicator (Biorupter Next Generation System – UCD300, Diagenode), with up to three runs of three cycles of 90 seconds each, until achieve a homogeneous solution.

For trehalose extraction, samples were incubated at 95 °C for 60 minutes and centrifuged for 15 minutes at 4 °C at 13200 rpm and 16100 g-force. We used 20 µl of the supernatant to determine trehalose concentration following the manufacturer protocol of the Megazyme trehalose kit (Megazyme, Bray, Ireland). This method relies on the difference in $NADPH^{+}$, before and after trehalose degradation by trehalase. Falcon Microtest 96 microplates were used for absorbance reads in an Infinite M200 Tecan spectrophotometer at 340 nm. For each 96-well plate (including two biological replicates each), we added eight blanks and two trehalose standards solution to calibrate and validate the reaction. After shaking three seconds and five-minute pause, sixteen measurements were taken per sample (4 x 4 matrix per well). Absorbance values were retrieved by i-control™ Microplate Reader Software by Tecan before and after trehalase addition. Calculation of trehalose concentration followed the manufacturer's instructions, accounting for egg volume and standard calibrations. According to the manufacturer's instructions, absorbance estimates below 0.1 are unreliable, which was the case for 13 of our 296 individual measures (*see* Table S1, S2 for details). We therefore excluded these replicates from statistical analysis, even though including them (setting estimates below 0.1 to an absorbance of 0.1) did not affect the outcome of the analysis (*see* supplementary data Table S3). To compare our estimates with other studies, we estimated dry weight and volume ratio per egg by using four replicates of 100 eggs each. We compared our trehalose estimates with another commonly used estimation method (high-performance liquid anion exchange chromatography) and were able to show that both methods reach the same results (Supplement section S5), providing us with confidence in the spectrophotometric method used here.

Data analysis

Data analysis was performed using the eight biological replicates, distributed evenly across four microtitre plates. We did not detect an effect of the microtitre plate (block-effect). The lme4

package was used to estimate the genotype variance component for trehalose content (lmer(conc_trehalose~(1|genotype)). Analysis of variance used summer-dry (Y/N) and winter-freeze (Y/N) as independent explanatory variables, and genotype variable as error, to test for differences in trehalose content (see Table 1). Our data followed normality and homoscedasticity assumptions. All analyses were performed with R (v 3.5) in R studio (v 1.2.5033). All material used is available in the supplement (Table S2, section S4).

Results

Mean percentage of trehalose in resting eggs was 10.55% of dry weight (stderr = 4.45). The among genotype variance component for these estimates was 14%. The mean percentage is similar to reports for some invertebrates with dry resting stages (nematodes (45), insects (46) and the lower crustacean *Artemia* (15% of dry weight)(22)). There was strong variation among and within genotypes (Fig. 2). Analysis of variance revealed a higher trehalose concentration (*p*-value = 0.001) in resting eggs from summer-dry habitat populations (Table1, Fig. 2). Factoring for winter-freezing did not reveal a significant difference, nor did the interaction between both factors (Table 1, Fig. 2).

Discussion

In this study, we show that trehalose concentration in resting stages varies among *Daphnia magna* genotypes and that this variation is partially influenced by the local habitat type. While previous studies focused on quantifying trehalose between species or between directly developing eggs and dormant stages (22,24,47), our study is the first to determine genetic variation within the same egg type of a species. Since all our genotypes were acclimated under similar laboratory conditions and produced resting eggs using eight independent replicates under the same conditions unrelated to their environment of origin, our results reflect genetic differences among the 37 genotypes studied here. This allows us to examine the evolution of trehalose concentration and test for its adaptive role across different environments. Our hypothesis—that trehalose concentration would be higher for genotypes from habitats with more severe conditions during diapause—was corroborated here, as genotypes from habitats with a high propensity for summer desiccation produced resting eggs containing about 20% more trehalose. This difference might be even greater when considering natural environmental triggers. The high (sometimes extreme) temperatures of the dry pond sediment (>50 °C (40)) constitute a severe stressful condition, requiring an efficient protection mechanism that trehalose is able to provide, as it fills the spaces left by water in the resting embryos' tissue with a glass-like structure and maintains the stability of cells and their contents (22,33).

In contrast to summer-dryness, we found no relation between trehalose concentration and winter-freezing. This finding is not very surprising because the resting stages in the pools we classified as winter-freezing pools may often not freeze solid; they generally only acquire ice at the surface. Except for very shallow pools such as Nordic rock pool habitats (40). Thus, resting eggs can often overwinter on the surface of the pond sediment without freezing stress. Also, the pools included in our study do not dry out in winter, so the severe combination of drying and freezing does not occur (5). A more detailed study with better data about local winter conditions may reveal an effect of winter harshness on trehalose concentration.

If trehalose is beneficial for the survival of resting eggs, one may expect it to be found equally in the resting eggs of all genotypes. Since this is not verified, trehalose production may be costly. A trade-off was found between storing and using energy metabolites for desiccation versus starvation stress in *Drosophila melanogaster* (48). Trehalose might also be involved in biotic interactions, as suggested in symbioses between higher plants and microorganisms (36,49), and in pathogenic interactions (50). An unexpected link may also exist between host trehalose concentrations and infection susceptibility, based on observations that only *D. magna* populations from summer-dry habitats are susceptible to the persistence of a virulent microsporidian parasite (41,51); however, it is unclear if elevated trehalose in resting eggs plays a causal role here. In interactions between *Plasmodium falciparum* and *Anopheles gambiae* mosquitoes, trehalose is likely a source of energy that enhances infection success (52). Further investigations in our study system might examine the relationship between host–parasite interaction and trehalose production.

Hengherr *et al*. (31) presented an estimate of trehalose in one genotype of *D. magna* resting eggs (0.5% of trehalose per dry weight), which is much lower than our estimates (about 10%). Since the quantification method differed between the two studies, we contacted the laboratory and conducted an experiment to quantify trehalose using duplicated samples of the same biological material, that were analysed by each laboratory following the methods previously applied to each study (this study and (31)). The two methods resulted in very similar trehalose estimates and where in accordance to values presented in our study (*see* Supplementary material section S5). The lower values presented in Hengherr et al. (31) might be explained by an extreme case of low trehalose concentration in resting eggs of a *D. magna* genotype from a summer-wet population.

Our study indicates that *Daphnia* resting eggs are locally adapted to the desiccation of their habitat in summer, allowing the species to inhabit a wider range of habitats and geographic areas, including very small water bodies that frequently dry up (53) and desert pools, where

water is only available for a limited period after rain fall (54). With ongoing climate change, an increased incidence of droughts across large geographic regions is predicted and can already be seen in the greater incidence of pools drying up in summer (55). *Daphnia magna* as an important component of many fresh- and brackish-water ecosystems will be strongly affected by such changes. Survival of local populations may critically depend on its ability to produce resting stages that can survive summer dryness. Understanding local adaptation to summer dryness is a first step to predict how species may evolve to cope with this aspect of climate change and provide insights on the future of abiotic and biotic interactions.

Data Accessibility

Data, additional analysis and R scripts for this study are available in the supplementary material section.

Authors' contribution

Both authors conceived the study, D.E. collected the *D. magna* genotypes, J.L.S collected the data, J.L.S. and D.E analysed the data. Both authors wrote the manuscript, agreed to be held accountable for the content therein and approved the final version of the manuscript.

Competing of interests

We declare we have no competing interests.

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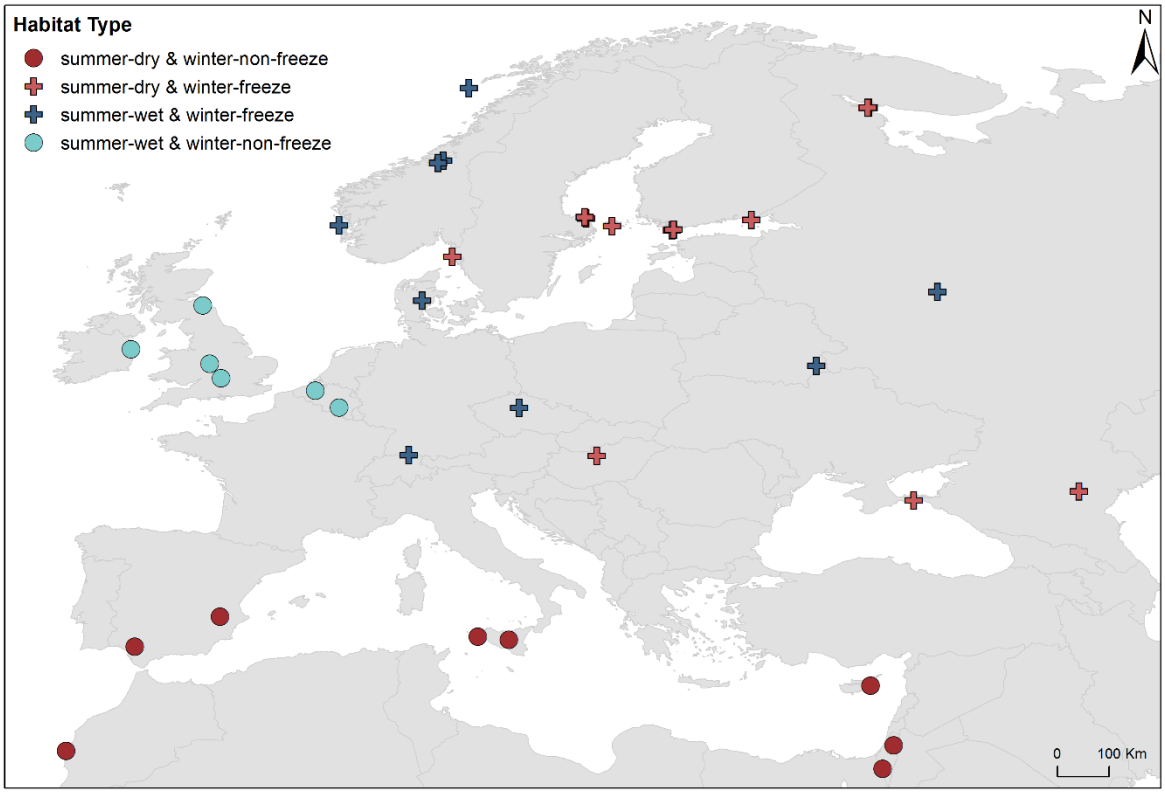
Figures and tables captions

Figure 1. Geographic distribution of sampling sites and their habitat types.

Figure 2. Average trehalose concentration for summer-dry (red) and summer-wet (blue) separated for habitat types (on the left) and for genotypes (on the right). Trehalose concentration is given as percentage of dry weight per resting egg. Box plots show median, first and third quartile. Whiskers extend to 1.5 times from the inter-quartile range upper and lower limits. The dots show datapoints beyond whiskers.

Table 1. Analysis of variance for the effect of habitat type and host genotype on trehalose concentration of *Daphnia magna* resting eggs. Significant p-values ($p \leq 0.001$) are shown in bold.

Figures



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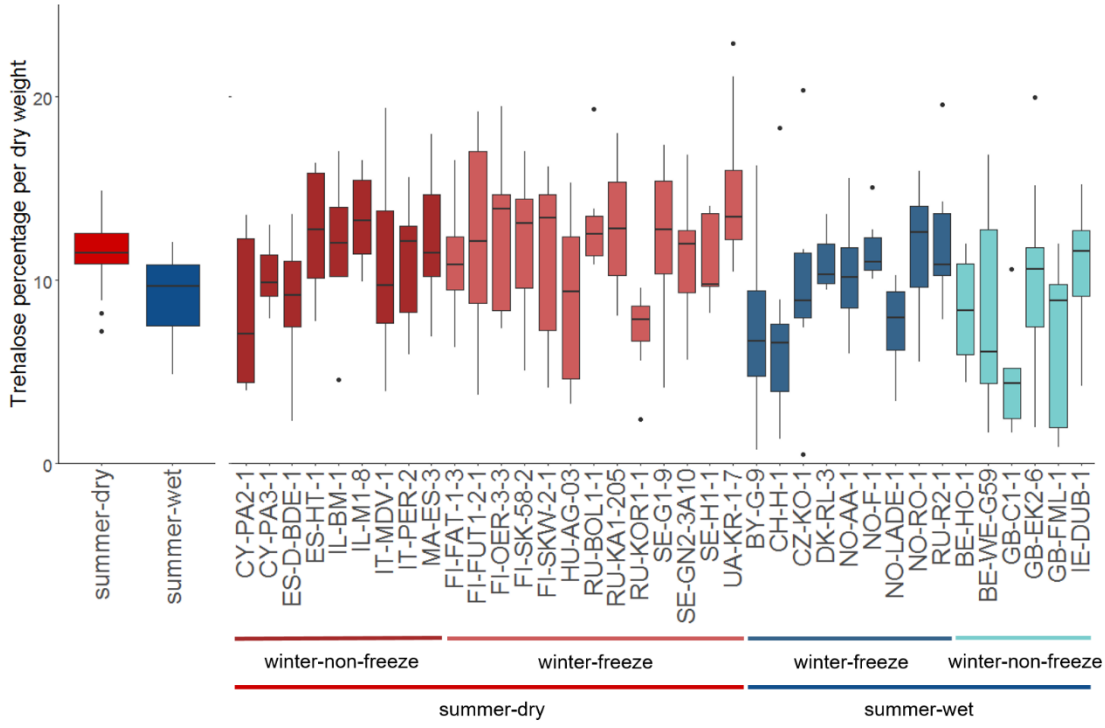

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Supplementary material

Trehalose provisioning in *Daphnia* resting stages reflects local adaptation to the harshness of diapause conditions

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Table S1. List of *Daphnia magna* genotypes, their origin (country, GPS coordinates), and the habitat type (summer-dry (sd) and winter-freeze (wf): Yes (Y) or No (N)). N1 and N2 are the number of replicates (N_1 excludes replicates with absorbance below 0.100; N_2 contains all replicates). Last two columns show mean percentage of trehalose per dry weight, excluding replicates with absorbance below 0.100 (tre_1) and including all replications (tre_2).

Genotype	Country	Latitude	Longitude	sd	wf	N_1	N_2	tre_1	tre_2
BE-HO-1	Belgium	50.1451	5.0771	N	N	8	8	8.32	8.32
BE-WE-G59	Belgium	51.0678	3.7736	N	N	8	8	8.11	8.11
BY-G-9	Belorussia	52.4215	31.0138	N	Y	7	8	7.40	7.24
CH-H-1	Switzerland	47.5578	8.8626	N	Y	8	8	7.02	7.02
CY-PA2-1	Cyprus	35.0328	33.9551	Y	N	6	8	8.18	7.84
CY-PA3-1	Cyprus	35.0341	33.9549	Y	N	7	8	10.23	9.50
CZ-KO-1	Czech-Republic	50.1254	14.8687	N	Y	8	8	9.63	9.63
DK-RL-3	Denmark	55.9642	9.5964	N	Y	6	8	10.95	9.00
ES-D-BDE1	Spain	37.1481	-6.0366	Y	N	8	8	9.00	9.00
ES-HT-1	Spain	38.7752	-1.4102	Y	N	8	8	12.66	12.66
FI-FAT-1-3	Finland	60.0217	19.9021	Y	Y	8	8	11.10	11.10
FI-FUT1-2-1	Finland	60.3471	27.4785	Y	Y	8	8	12.08	12.08
FI-OER-3-3	Finland	59.7886	23.1741	Y	Y	8	8	12.64	12.64
FI-SK-58-2	Finland	59.833	23.2574	Y	Y	8	8	11.76	11.76
FI-SKW-2-1	Finland	59.833	23.2563	Y	Y	8	8	11.13	11.13
GB-C1-1	Great-Britain	51.7344	-1.3363	N	N	5	8	4.83	4.70
GB-EK2-6	Great-Britain	55.6977	-2.3434	N	N	8	8	10.33	10.33
GB-FML-1	Great-Britain	52.5311	-1.9559	N	N	8	8	6.78	6.78
HU-AG-03	Hungary	47.5146	19.0813	Y	Y	8	8	8.88	8.88
IE-DUB-1	Ireland	53.3267	-6.2341	N	N	7	8	10.64	9.92
IL-BM-1	Israel	30.5113	34.6121	Y	N	8	8	11.97	11.97
IL-M1-8	Israel	31.7782	35.2206	Y	N	8	8	13.37	13.37
IT-MDV-1	Italy	37.6855	12.6175	Y	N	8	8	11.10	11.10
IT-PER-2	Italy	37.5192	14.3073	Y	N	7	8	10.84	10.15
MA-ES-3	Morocco	31.4907	-9.7644	Y	N	7	8	12.27	11.80
NO-AA-1	Norway	60.051	5.0744	N	Y	8	8	10.29	10.29
NO-F-1	Norway	63.5877	10.729	N	Y	8	8	11.60	11.60
NO-LADE-1	Norway	63.449	10.4529	N	Y	8	8	7.52	7.52
NO-RO-1	Norway	67.5274	12.1268	N	Y	8	8	11.59	11.59
RU-BOL1-1	Russia	66.4497	33.8567	Y	Y	8	8	13.08	13.08
RU-KA1-205	Russia	45.5994	45.2975	Y	Y	8	8	12.95	12.95
RU-KOR1-1	Russia	66.4519	33.799	Y	Y	7	8	7.17	6.49
RU-R2-1	Russia	56.425	37.6027	N	Y	8	8	12.04	12.04
SE-G1-9	Sweden	60.4217	18.5102	Y	Y	8	8	12.22	12.22
SE-GN2-3A10	Sweden	60.4971	18.4316	Y	Y	8	8	11.19	11.19
SE-H1-1	Sweden	58.3423	11.218	Y	Y	8	8	11.01	11.01
UA-KR-1-7	Ukraine	45.0937	36.3107	Y	Y	8	8	14.86	14.86

Table S2. List of individual *Daphnia magna* (replicates) used for each genotype in this study. Detailed data for each replicate (rep), with description of habitat type, specifically summer-dry (sd) and winter-freeze (sd): Yes (Y) or No (N); sample volume in mg (vsample), and trehalose percentage per dry weight (conc_trehalose). Biological sample replicates with an asterisk (*) had absorbance below 0.1 and were excluded from the main analysis.

genotype	rep	sd	wf	vsample	conc_trehalose
BE-HO-1	1	N	N	0.062	5.85
BE-HO-1	2	N	N	0.086	10.88
BE-HO-1	3	N	N	0.065	5.92
BE-HO-1	4	N	N	0.058	10.67
BE-HO-1	5	N	N	0.069	6.00
BE-HO-1	6	N	N	0.063	10.88
BE-HO-1	7	N	N	0.068	4.41
BE-HO-1	8	N	N	0.068	11.93
BE-WE-G59	1	N	N	0.060	1.67
BE-WE-G59	2	N	N	0.058	16.82
BE-WE-G59	3	N	N	0.054	5.75
BE-WE-G59	4	N	N	0.053	13.02
BE-WE-G59	5	N	N	0.048	4.44
BE-WE-G59	6	N	N	0.059	6.37
BE-WE-G59	7	N	N	0.068	4.13
BE-WE-G59	8	N	N	0.062	12.66
BY-G-9	1*	N	Y	0.059	6.11
BY-G-9	2	N	Y	0.069	7.52
BY-G-9	3	N	Y	0.054	6.44
BY-G-9	4	N	Y	0.067	6.66
BY-G-9	5	N	Y	0.058	0.73
BY-G-9	6	N	Y	0.064	11.28
BY-G-9	7	N	Y	0.063	3.00
BY-G-9	8	N	Y	0.068	16.19
CH-H-1	1	N	Y	0.063	3.25
CH-H-1	2	N	Y	0.077	18.29
CH-H-1	3	N	Y	0.067	6.36
CH-H-1	4	N	Y	0.078	6.73
CH-H-1	5	N	Y	0.078	1.31
CH-H-1	6	N	Y	0.070	8.91
CH-H-1	7	N	Y	0.071	4.14
CH-H-1	8	N	Y	0.077	7.15
CY-PA2-1	1*	Y	N	0.061	5.89
CY-PA2-1	2	Y	N	0.053	13.34
CY-PA2-1	3	Y	N	0.047	5.17
CY-PA2-1	4	Y	N	0.059	4.15
CY-PA2-1	5	Y	N	0.047	3.95

CY-PA2-1	6	Y	N	0.044	8.95
CY-PA2-1	7*	Y	N	0.046	7.76
CY-PA2-1	8	Y	N	0.044	13.53
CY-PA3-1	1	Y	N	0.056	9.35
CY-PA3-1	2	Y	N	0.058	12.73
CY-PA3-1	3	Y	N	0.059	8.83
CY-PA3-1	4	Y	N	0.055	9.97
CY-PA3-1	5*	Y	N	0.058	4.44
CY-PA3-1	6	Y	N	0.058	9.85
CY-PA3-1	7	Y	N	0.054	7.90
CY-PA3-1	8	Y	N	0.055	12.96
CZ-KO-1	1	N	Y	0.086	8.13
CZ-KO-1	2	N	Y	0.064	20.35
CZ-KO-1	3	N	Y	0.075	7.38
CZ-KO-1	4	N	Y	0.079	9.03
CZ-KO-1	5	N	Y	0.074	0.47
CZ-KO-1	6	N	Y	0.071	8.69
CZ-KO-1	7	N	Y	0.065	11.63
CZ-KO-1	8	N	Y	0.073	11.38
DK-RL-3	1	N	Y	0.079	12.43
DK-RL-3	2	N	Y	0.075	13.55
DK-RL-3	3	N	Y	0.079	9.68
DK-RL-3	4	N	Y	0.075	10.43
DK-RL-3	5*	N	Y	0.080	3.20
DK-RL-3	6*	N	Y	0.081	3.15
DK-RL-3	7	N	Y	0.085	9.46
DK-RL-3	8	N	Y	0.080	10.14
ES-D-BDE-1	1	Y	N	0.049	2.27
ES-D-BDE-1	2	Y	N	0.054	13.57
ES-D-BDE-1	3	Y	N	0.047	8.82
ES-D-BDE-1	4	Y	N	0.047	9.52
ES-D-BDE-1	5	Y	N	0.054	7.25
ES-D-BDE-1	6	Y	N	0.063	7.53
ES-D-BDE-1	7	Y	N	0.055	10.47
ES-D-BDE-1	8	Y	N	0.047	12.55
ES-HT-1	1	Y	N	0.050	9.79
ES-HT-1	2	Y	N	0.044	16.02
ES-HT-1	3	Y	N	0.061	7.72
ES-HT-1	4	Y	N	0.054	11.90
ES-HT-1	5	Y	N	0.053	10.18
ES-HT-1	6	Y	N	0.047	15.79
ES-HT-1	7	Y	N	0.048	16.38
ES-HT-1	8	Y	N	0.059	13.54
FI-FAT-1-3	1	Y	Y	0.065	9.97
FI-FAT-1-3	2	Y	Y	0.056	16.49

FI-FAT-1-3	3	Y	Y	0.065	6.31
FI-FAT-1-3	4	Y	Y	0.063	9.62
FI-FAT-1-3	5	Y	Y	0.065	8.94
FI-FAT-1-3	6	Y	Y	0.073	11.77
FI-FAT-1-3	7	Y	Y	0.061	11.63
FI-FAT-1-3	8	Y	Y	0.067	14.08
FI-FUT1-2-1	1	Y	Y	0.072	11.42
FI-FUT1-2-1	2	Y	Y	0.063	16.92
FI-FUT1-2-1	3	Y	Y	0.064	3.69
FI-FUT1-2-1	4	Y	Y	0.067	12.74
FI-FUT1-2-1	5	Y	Y	0.061	5.87
FI-FUT1-2-1	6	Y	Y	0.060	9.68
FI-FUT1-2-1	7	Y	Y	0.061	19.16
FI-FUT1-2-1	8	Y	Y	0.065	17.16
FI-OER-3-3	1	Y	Y	0.058	13.75
FI-OER-3-3	2	Y	Y	0.053	19.46
FI-OER-3-3	3	Y	Y	0.050	7.36
FI-OER-3-3	4	Y	Y	0.058	14.00
FI-OER-3-3	5	Y	Y	0.060	7.95
FI-OER-3-3	6	Y	Y	0.055	15.98
FI-OER-3-3	7	Y	Y	0.063	8.44
FI-OER-3-3	8	Y	Y	0.065	14.20
FI-SK-58-2	1	Y	Y	0.062	14.38
FI-SK-58-2	2	Y	Y	0.067	11.96
FI-SK-58-2	3	Y	Y	0.067	10.46
FI-SK-58-2	4	Y	Y	0.066	17.01
FI-SK-58-2	5	Y	Y	0.075	5.01
FI-SK-58-2	6	Y	Y	0.070	14.38
FI-SK-58-2	7	Y	Y	0.069	6.71
FI-SK-58-2	8	Y	Y	0.062	14.19
FI-SKW-2-1	1	Y	Y	0.060	14.53
FI-SKW-2-1	2	Y	Y	0.071	16.18
FI-SKW-2-1	3	Y	Y	0.067	4.33
FI-SKW-2-1	4	Y	Y	0.059	12.57
FI-SKW-2-1	5	Y	Y	0.061	4.10
FI-SKW-2-1	6	Y	Y	0.066	8.19
FI-SKW-2-1	7	Y	Y	0.059	14.18
FI-SKW-2-1	8	Y	Y	0.057	14.94
GB-C1-1	1	N	N	0.059	4.36
GB-C1-1	2	N	N	0.074	5.16
GB-C1-1	3	N	N	0.067	1.63
GB-C1-1	4	N	N	0.061	2.42
GB-C1-1	5*	N	N	0.058	4.44
GB-C1-1	6*	N	N	0.078	3.30
GB-C1-1	7*	N	N	0.062	5.69

GB-C1-1	8	N	N	0.046	10.59
GB-EK2-6	1	N	N	0.059	10.63
GB-EK2-6	2	N	N	0.068	19.94
GB-EK2-6	3	N	N	0.062	7.89
GB-EK2-6	4	N	N	0.064	15.13
GB-EK2-6	5	N	N	0.069	1.92
GB-EK2-6	6	N	N	0.066	10.59
GB-EK2-6	7	N	N	0.071	6.01
GB-EK2-6	8	N	N	0.066	10.56
GB-FML-1	1	N	N	0.079	1.99
GB-FML-1	2	N	N	0.065	10.42
GB-FML-1	3	N	N	0.103	1.86
GB-FML-1	4	N	N	0.066	9.48
GB-FML-1	5	N	N	0.069	0.85
GB-FML-1	6	N	N	0.073	8.81
GB-FML-1	7	N	N	0.067	8.89
GB-FML-1	8	N	N	0.061	11.93
HU-AG-03	1	Y	Y	0.056	3.20
HU-AG-03	2	Y	Y	0.058	15.30
HU-AG-03	3	Y	Y	0.054	4.85
HU-AG-03	4	Y	Y	0.052	8.61
HU-AG-03	5	Y	Y	0.055	3.83
HU-AG-03	6	Y	Y	0.056	12.07
HU-AG-03	7	Y	Y	0.060	10.06
HU-AG-03	8	Y	Y	0.047	13.11
IE-DUB-1	1	N	N	0.050	4.19
IE-DUB-1	2	N	N	0.041	11.54
IE-DUB-1	3	N	N	0.045	10.65
IE-DUB-1	4	N	N	0.057	12.72
IE-DUB-1	5*	N	N	0.052	4.90
IE-DUB-1	6	N	N	0.045	15.17
IE-DUB-1	7	N	N	0.051	7.55
IE-DUB-1	8	N	N	0.044	12.67
IL-BM-1	1	Y	N	0.043	12.98
IL-BM-1	2	Y	N	0.044	16.86
IL-BM-1	3	Y	N	0.048	4.55
IL-BM-1	4	Y	N	0.046	11.93
IL-BM-1	5	Y	N	0.042	10.23
IL-BM-1	6	Y	N	0.047	12.09
IL-BM-1	7	Y	N	0.053	10.11
IL-BM-1	8	Y	N	0.041	17.01
IL-M1-8	1	Y	N	0.051	16.50
IL-M1-8	2	Y	N	0.063	14.01
IL-M1-8	3	Y	N	0.055	11.48
IL-M1-8	4	Y	N	0.055	12.39

IL-M1-8	5	Y	N	0.058	9.87
IL-M1-8	6	Y	N	0.052	15.08
IL-M1-8	7	Y	N	0.060	11.15
IL-M1-8	8	Y	N	0.061	16.44
IT-MDV-1	1	Y	N	0.063	11.97
IT-MDV-1	2	Y	N	0.054	19.37
IT-MDV-1	3	Y	N	0.055	8.86
IT-MDV-1	4	Y	N	0.052	19.09
IT-MDV-1	5	Y	N	0.055	7.45
IT-MDV-1	6	Y	N	0.057	10.50
IT-MDV-1	7	Y	N	0.064	3.91
IT-MDV-1	8	Y	N	0.061	7.68
IT-PER-2	1	Y	N	0.058	12.91
IT-PER-2	2	Y	N	0.051	15.55
IT-PER-2	3	Y	N	0.063	6.86
IT-PER-2	4	Y	N	0.061	12.94
IT-PER-2	5	Y	N	0.051	5.91
IT-PER-2	6	Y	N	0.061	9.62
IT-PER-2	7*	Y	N	0.067	5.31
IT-PER-2	8	Y	N	0.064	12.11
MA-ES-3	1	Y	N	0.038	6.91
MA-ES-3	2	Y	N	0.037	16.72
MA-ES-3	3	Y	N	0.037	17.93
MA-ES-3	4	Y	N	0.036	11.45
MA-ES-3	5	Y	N	0.039	10.02
MA-ES-3	6	Y	N	0.039	12.54
MA-ES-3	7*	Y	N	0.042	8.50
MA-ES-3	8	Y	N	0.042	10.34
NO-AA-1	1	N	Y	0.069	15.53
NO-AA-1	2	N	Y	0.062	11.56
NO-AA-1	3	N	Y	0.073	8.11
NO-AA-1	4	N	Y	0.070	11.21
NO-AA-1	5	N	Y	0.060	5.95
NO-AA-1	6	N	Y	0.058	12.31
NO-AA-1	7	N	Y	0.084	8.59
NO-AA-1	8	N	Y	0.073	9.04
NO-F-1	1	N	Y	0.051	10.61
NO-F-1	2	N	Y	0.052	12.12
NO-F-1	3	N	Y	0.054	11.09
NO-F-1	4	N	Y	0.048	10.33
NO-F-1	5	N	Y	0.055	12.72
NO-F-1	6	N	Y	0.050	10.84
NO-F-1	7	N	Y	0.050	10.03
NO-F-1	8	N	Y	0.054	15.01
NO-LADE-1	1	N	Y	0.074	5.02

NO-LADE-1	2	N	Y	0.054	9.10
NO-LADE-1	3	N	Y	0.064	10.01
NO-LADE-1	4	N	Y	0.062	8.18
NO-LADE-1	5	N	Y	0.068	3.35
NO-LADE-1	6	N	Y	0.077	7.66
NO-LADE-1	7	N	Y	0.061	6.57
NO-LADE-1	8	N	Y	0.071	10.25
NO-RO-1	1	N	Y	0.061	13.87
NO-RO-1	2	N	Y	0.063	14.39
NO-RO-1	3	N	Y	0.060	7.49
NO-RO-1	4	N	Y	0.065	10.33
NO-RO-1	5	N	Y	0.063	5.53
NO-RO-1	6	N	Y	0.061	13.12
NO-RO-1	7	N	Y	0.064	12.08
NO-RO-1	8	N	Y	0.063	15.92
RU-BOL1-1	1	Y	Y	0.061	13.34
RU-BOL1-1	2	Y	Y	0.073	12.11
RU-BOL1-1	3	Y	Y	0.067	13.86
RU-BOL1-1	4	Y	Y	0.067	12.87
RU-BOL1-1	5	Y	Y	0.070	11.45
RU-BOL1-1	6	Y	Y	0.056	10.91
RU-BOL1-1	7	Y	Y	0.069	10.82
RU-BOL1-1	8	Y	Y	0.065	19.30
RU-KA1-205	1	Y	Y	0.070	11.30
RU-KA1-205	2	Y	Y	0.069	18.00
RU-KA1-205	3	Y	Y	0.067	14.90
RU-KA1-205	4	Y	Y	0.075	14.25
RU-KA1-205	5	Y	Y	0.064	8.03
RU-KA1-205	6	Y	Y	0.066	10.28
RU-KA1-205	7	Y	Y	0.064	16.66
RU-KA1-205	8	Y	Y	0.056	10.17
RU-KOR1-1	1	Y	Y	0.058	9.31
RU-KOR1-1	2	Y	Y	0.052	7.86
RU-KOR1-1	3	Y	Y	0.167	1.76
RU-KOR1-1	4*	Y	Y	0.048	7.84
RU-KOR1-1	5	Y	Y	0.043	7.70
RU-KOR1-1	6	Y	Y	0.052	2.39
RU-KOR1-1	7	Y	Y	0.048	9.54
RU-KOR1-1	8	Y	Y	0.040	5.56
RU-R2-1	1	N	Y	0.065	10.84
RU-R2-1	2	N	Y	0.069	13.43
RU-R2-1	3	N	Y	0.061	9.10
RU-R2-1	4	N	Y	0.058	10.75
RU-R2-1	5	N	Y	0.074	7.84
RU-R2-1	6	N	Y	0.063	10.61

RU-R2-1	7	N	Y	0.063	19.54
RU-R2-1	8	N	Y	0.063	14.23
SE-G1-9	1	Y	Y	0.067	4.11
SE-G1-9	2	Y	Y	0.050	17.34
SE-G1-9	3	Y	Y	0.071	10.73
SE-G1-9	4	Y	Y	0.063	9.10
SE-G1-9	5	Y	Y	0.061	12.23
SE-G1-9	6	Y	Y	0.053	15.30
SE-G1-9	7	Y	Y	0.063	13.28
SE-G1-9	8	Y	Y	0.058	15.68
SE-GN2-3A10	1	Y	Y	0.050	12.16
SE-GN2-3A10	2	Y	Y	0.043	11.77
SE-GN2-3A10	3	Y	Y	0.039	9.79
SE-GN2-3A10	4	Y	Y	0.043	13.08
SE-GN2-3A10	5	Y	Y	0.056	5.64
SE-GN2-3A10	6	Y	Y	0.050	12.55
SE-GN2-3A10	7	Y	Y	0.045	7.77
SE-GN2-3A10	8	Y	Y	0.050	16.79
SE-H1-1	1	Y	Y	0.067	13.57
SE-H1-1	2	Y	Y	0.060	9.72
SE-H1-1	3	Y	Y	0.067	8.16
SE-H1-1	4	Y	Y	0.063	13.66
SE-H1-1	5	Y	Y	0.056	9.61
SE-H1-1	6	Y	Y	0.054	13.98
SE-H1-1	7	Y	Y	0.056	9.73
SE-H1-1	8	Y	Y	0.060	9.66
UA-KR-1-7	1	Y	Y	0.052	10.41
UA-KR-1-7	2	Y	Y	0.058	21.05
UA-KR-1-7	3	Y	Y	0.055	10.74
UA-KR-1-7	4	Y	Y	0.055	14.28
UA-KR-1-7	5	Y	Y	0.055	13.64
UA-KR-1-7	6	Y	Y	0.047	13.21
UA-KR-1-7	7	Y	Y	0.058	12.68
UA-KR-1-7	8	Y	Y	0.048	22.90

Table S3. Analysis of variance for the effect of habitat type and host genotype on trehalose concentration of *Daphnia magna* resting eggs, including all samples. Significant *p*-values are shown in bold ($p \leq 0.01$).

Factor	*Df*	*Mean of squares*	*F value*	*P value*
Summer-dry	1	370.6	11.74	**0.0016**
Winter-freeze	1	73.3	2.32	0.137
Summer-dry: Winter-freeze	1	12.4	0.39	0.534
Residuals	33	31.6	-	-
Error: Genotype	259	15.76		

Section S4 – R scripts used for the analysis of this study

Analysis of variance

```
# R packages used in this analysis

>library(lme4)


# Input data for main analysis

>dat1 <-read.table("input_file.csv", sep = ";",  head=TRUE)


# Analysis of variance

> variance_analysis<-aov(dat1$conc_trehalose ~ dat1$sd * dat1$wf + Error(dat1$genotype))

>summary(variance_analysis)
```

Analysis of variances and tests for checking normality of data and homogeneity of variances were repeated by using all data entries including ones with absorbance below 0.1, following the same methods.

```
#Normality test – Shapiro test – Normality of data residuals
> model<-lm(dat1$conc_trehalose~ dat1$sd * dat1$wf + (dat1$genotype), data=dat1)

>res<-resid(model)

>plot(fitted(model), res)

>qqnorm(res)

>hist(res)

>plot(density(res))

>shapiro.test(res)


#Bartlett's test – Homogeneity of variances

>bartlett.test(conc_trehalose ~ interaction(sd,wf,genotype), data=dat1)
```

Analysis of variance of genotypes component

```
#R packages used in this analysis
```

```
>library(lme4)

# Analysis of variance of genotype component

>genotype_variance<- lmer(conc_trehalose ~ (1|genotype), data=dat1) #genotype is the random variable

>summary(genotype_variance)


# Calculation of genotype variance component

>genotype_variance_perc<- “variance of genotype”/( “variance of genotype ”+”variance of residual”) * 100

#in this case the genotype variance percentage value was obtained by the following

>genotype_variance_perc<- 2.635/(2.635+ 15.739) *100
```

Section S5 – Comparison of methods for trehalose quantification in *Daphnia magna* resting eggs.

This section profited from the contributions of Ralph O. Schill and Arnd G. Heyer from the University of Stuttgart, Germany.

Overview: Estimations of trehalose concentration presented in this study largely diverged from the ones estimated in Hengherr *et al.* (2011). Our main study reported an average of 10.55 % of trehalose per *D. magna* resting egg dry weight, whereas Hengherr *et al*. (2011) reported 0.5 % of trehalose per dry weight. The main difference between the two studies is the trehalose quantification method. In our study we used a calorimetric method following the Megazyme trehalose kit (Megazyme Bray, Ireland). Hengherr *et al*. (2011) used a high-performance liquid anion exchange chromatography (HPAEC) using a CarboPac PA-1 collumn on a Dionex DX-500 gradient chromatography system coupled with pulsed amperometric detection by a gold electrode (Dionex, Sunnyvale, CA).

Here we report on a comparison of the estimations of trehalose concentration in *Daphnia magna* resting eggs based on the two different quantification methods (our main study and Hengherr *et al.* 2011). The method applied in our study was run in the University of Basel and the method applied in Hengherr *et al*. (2011) was run in the University of Stuttgart. The samples were produced in duplicate at the University of Basel and the same biological material was split into two, in order to be analysed separately by the two distinct methods in the two research groups.

Methods: Four sets of thirty *Daphnia magna* resting eggs were decapsulated from the ephippial shell providing four samples. For each samples the total egg volume was calculated and a samples solution of extracted trehalose was prepared following the same methodology implemented both in our study and Hengherr *et al*. 2011. Furthermore, three blanks samples (ultrapure water) and three samples with trehalose of known concentration (180.9 mg/L) were produced. At this stage, each sample was separated into two portions of 120 µL (to be used in HPAEC method) and 20 µL (to be used in Megazyme trehalose kit method). Samples were frozen until measurements were performed (which took place in the two laboratories within three days). Sample codes were replaced with random numbers (Table S5.1).

Trehalose was quantified in each of the duplicated samples by both High-performance liquid anion exchange chromatography method in the University of Stuttgart, as in Hengherr *et al.* (2011), and trehalose Megazyme kit (Megazyme, Bray, Ireland), in the University of Basel, as described in the main experiment of our study.

Table S5.1 – Description of samples used in this experiment. Note that egg-samples B and A are from a different genetic *Daphnia magna* background than samples C and D, and are expected to differ to some degree.

Code	Sample	Origin of Daphnia eggs
B1	Sample B	SE-H1-4
B2	Blank 1	-
B3	Sample A	SE-H1-4
B4	Trehalose 1	-
B5	Blank 2	-
B6	Sample C	FI-FUT1-2-1
B7	Blank 3	-
B8	Sample D	FI-FUT1-2-1
B9	Trehalose 2	-
B10	Trehalose 3	-

Results: For both quantification methods, blank samples B2, B5 and B7 showed no trehalose, as expected. The quantities obtained by the two methods for the egg samples (trehalose concentration (g/L and mol/L)) were very similar. Specifically, the average trehalose concentration present on egg samples (B1, B3, B6 and B8) were of $4.2x10^{-4}$ mol/L for HPAEC method and $3.8x10^{-4}$ mol/L Megazyme trehalose kit method, resulting in, on average, about 10 % higher estimates by HPAEC method. In regard to the trehalose standard samples (B4, B9 and B10), average estimation was $5.97x10^{-4}$ mol/L and $4.78x10^{-4}$ mol/L, for each HPAEC and Megazyme trehalose kit method respectively. The trehalose samples comprised a known trehalose solution of 180.9 mg/L concentration (i.e. $4.8x10^{-4}$ mol/L). The HPAEC method overestimated this amount by 24 % and Megazyme trehalose kit method estimations were very close to the expected. However, the difference between the two methods was not significant (paired T-test: t=1.98, df=2, *p*-value=0.19).

Additionally, for each egg samples, trehalose per egg (g), trehalose per wet weight of egg (g/g) and trehalose per dry weight of egg (g/g) was calculated (*see* Table S5.2). Trehalose per wet weight was measured based on the estimation of eggs volume included in each sample, assuming a density of 1 (equal to water). Trehalose per dry weight was measured considering previous estimations in our laboratory of the ration between wet and dry egg for *Daphnia magna* resting eggs (i.e. estimations based on four samples of 100 eggs each). Mean of wet weight and dry weight per egg was estimated as 15.0 µg and 6.735 µg, respectively (thus, ration between egg dry and wet weight is 0.425). For comparison, the dry weight reported in Hengherr *et al.* (2011) is 7.39 µg. On average trehalose per egg dry weight was estimated as 10.7 % and 9.8 %, respectively for HPAEC and Megazyme trehalose kit methods (Table S5.2). This difference is not significant (paired T-test: t=1.85, df=3, *p*-value=0.16).

Table S5.2 – Estimations of trehalose per egg (g), trehalose per egg wet and dry weight (g/g) for the trehalose quantification methods HPAEC and Megazyme trehalose kit performed in this study.

Samples		Egg weight		High-performance liquid anion exchange chromatography (Stuttgart)			Megazyme trehalose kit (Basel)		
Code	Sample	eggs wet weight [mg]	eggs dry weight [mg]	Trehalose per egg [g]	Trehalose per egg wet weight [g/g]	Trehalose per egg dry weight [g/g]	Trehalose per egg [g]	Trehalose per egg wet weight [g/g]	Trehalose per egg dry weight [g/g]
B1	Sample B	0.56	0.24	6.86E-07	0.036	0.086	6.21E-07	0.033	0.078
B3	Sample A	0.58	0.24	6.60E-07	0.034	0.081	6.53E-07	0.034	0.080
B6	Sample C	0.50	0.21	9.00E-07	0.054	0.128	7.31E-07	0.044	0.103
B8	Sample D	0.48	0.20	9.09E-07	0.057	0.134	8.75E-07	0.055	0.129
Average		0.53	0.23	7.89E-07	0.043	0.107	7.20E-07	0.042	0.098

Discussion: The results of this experiment showed that the two methods used to quantify trehalose resulted in similar estimates and that the amounts of trehalose provisioning in *D. magna* resting eggs are in accordance to the estimates of our main study.

The discrepancy between the reported values in our main study and in Hengherr *et al.* (2011) cannot be explained by these new quantifications, because this discrepancy seems neither be attributable to a difference among the two research groups nor to a difference in the quantification methods applied. Examination of the data presented in Hengherr *et al.* (2011) shows that egg dry weight is roughly similar between them and this study (7.39 µg and 6.375 µg, respectively). *Daphnia magna* resting eggs can vary by factor two in volume, even for eggs from the same female. Also, genotypes differ in the size of egg they produce. In addition, the Hengherr *et al.* (2011) reported hatching rates of previously dried resting eggs of 14 %, which fits the lower end of hatching rates reported otherwise. We conclude that, in regard to these parameters the *D. magna* genotype used by Hengherr *et al.* (2011) is not unusual. Estimations of trehalose content on *D. magna* resting eggs remain distinct between the two studies. An explanation might be due to an extreme case of biological material used by Hengherr *et al.* (2011).

The trehalose level of the resting stages of *Daphnia magna* reported in our study are in the general range observed for other organisms, for example *Polypedilum vanderplanki* (insecta), *Artemia* (lower Crustacea) and nematods, and where trehalose levels were reported to be between 10 % and 20 % of dry weight (Clegg 1965; Madin and Crowe 1975; Watanabe *et al.* 2002).

References:

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Tables

Table 1. Analysis of variance for the effect of habitat type and host genotype on trehalose concentration of *Daphnia magna* resting eggs. Significant p-values ($p \leq 0.001$) are shown in bold.

Factor	*Df*	*Mean of squares*	*F value*	*P value*
Summer-dry	1	327.3	12.0	**0.001**
Winter-freeze	1	47.0	1.73	0.198
Summer-dry: Winter-freeze	1	16.5	0.61	0.442
Residuals	33	27.3		
Error: Genotype	246	15.69		