Supplementary Information for

Combined metabolome and transcriptome analysis reveals key components of complete desiccation tolerance in an anhydrobiotic insect Polypedilum vanderplanki

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In this study, we compared the effect of the desiccation-rehydration process on the content and proportions of metabolites in *P. vanderplanki* larvae and its non-anhydrobiotic sister species *P. nubifer*. Based on this comparison with the desiccation-sensitive species, *P. nubifer*, we conclude that the dynamics of the metabolome are tightly linked to anhydrobiosis, and likely to be one of the key factors required for extreme tolerance.

According to the Human Metabolome Database 4.0 (HMDB; www.hmdb.ca) hierarchical classification (Fig. S1), most of the identified metabolites fell into the “organic acids and derivatives” and “nucleosides, nucleotides, and analogues” categories.

**Fig. S1.** Distribution of metabolite groups identified in *P. vanderplanki* larvae according to HMDB hierarchical classification. Some were not present in the HMDB and were marked “not specified”.

At all time points, the most prevalent metabolites were amino acids (especially glutamine, glutamic acid and arginine), citric acid and the adenosine phosphates (ATP, ADP, AMP). A high content of malic acid, succinic acid, lactic acid, gamma-aminobutyric acid (GABA) and glucose 6-phosphate was also observed.

Besides the presence and concentration level of metabolites, their ratios at different stages of anhydrobiosis can better illustrate metabolic changes during the desiccation-rehydration cycle (Table S1). In the metabolome of desiccated larvae (D48), the levels of 112 compounds increased compared to hydrated larvae (D0), while 108 decreased and 46 did not change significantly. Among the metabolites of rehydrated larvae (R3), 113 compounds increased in level compared to desiccated larvae (D48), while 113 decreased and 40 did not change. The biggest increase in desiccated larvae (D48/D0) was for trehalose-6-phosphate, whose concentration was 47 times higher than in hydrated larvae. In contrast, three hours rehydration (R3/D48) resulted in the biggest upward shift in content for glycolysis/gluconeogenesis intermediates: pyruvic acid (63...
lactic acid (50 ↑), glucose 6-phosphate (48 ↑), fructose 6-phosphate (31 ↑), etc. Comparison of metabolite patterns between desiccated (D48/D0) or rehydrated larvae (R3/D0) showed a significant accumulation of kynurenine and intermediates of the urea cycle during anhydrobiosis, as well as the accumulation of different sugar phosphates. In contrast, the biggest decrease in metabolite content during desiccation (D48/D0) was observed among citric acid cycle (CAC) and some glycolysis intermediates: fumaric acid (25 ↓), phosphoenolpyruvic acid (20 ↓), malic acid (20 ↓), 2-phosphoglyceric acid (11 ↓), etc.

Comparative metabolomic profiling of *P. vanderplanki* and *P. nubifer* larvae identified 272 metabolites (full list of metabolites in Table S2) across the several points corresponding to the time in hours from the beginning of desiccation process: 0 h (D0, control); 12 h (D12); 24 h (D24); 36 h (D36); 48 h (D48). For anhydrobiotic *P. vanderplanki* larvae, sample preparation was continued with rehydration course: 1 h (R1), 3 h (R3), 12 h (R12) and 24 h (R24) after adding water, but also with wet larvae and dry anhydrobiotic larvae as controls. Thus, samples corresponding to wet larvae (=D0) and to dry larvae (=D48) were obtained in duplicate from desiccation process and rehydration process experiments. For non-anhydrobiotic *P. nubifer* chironomid sample preparation was stopped after 24 hours of desiccation, because of larvae’s mortality. In contrast to sweeping changes in the metabolome of *P. vanderplanki* during anhydrobiosis, most metabolites in desiccation-sensitive *P. nubifer* showed a tendency to decrease in content or did not change significantly. Some intermediates of ammonia recycling and fatty acid metabolism represented minor exceptions. Presumably, such differences in the metabolome response to dehydration arise from the ability of *P. vanderplanki* larvae to undergo anhydrobiosis, enabling its corresponding stress resistance, which are lacking in *P. nubifer* midges.
Carbohydrate metabolism

In the metabolome of desiccated larvae, the precursor of trehalose biosynthesis – trehalose-6-phosphate (T6P), was the only sugar whose level increased (47-fold compared to hydrated larvae). The accumulation of T6P was detected as soon as 12h after the beginning of desiccation and was accelerated at the very end of anhydrobiosis induction, between 36h and 48h of desiccation (Fig. S2). T6P degradation occurred rapidly during the first 3h after rehydration.

![Fig. S2. Changes in trehalose-6-phosphate and glucose-6-phosphate levels shown by comparative metabolic profiling of P. vanderplanki (red circles) and P. nubifer (green diamonds) larvae. Corresponding to changes in metabolite content, Y-axes represent arbitrary units based on the relative metabolite peak area. Standard deviation is shown for D0 and D48 samples obtained in duplicate for P. vanderplanki.](image)

On the other hand, glucose-6-phosphate (G6P) level, which is an intermediate metabolite for trehalose synthesis, decreased between 36h and 48h of desiccation. Its level was restored rapidly during the first hours following rehydration, reflecting the degradation of trehalose to glucose (Fig. S2).

In the desiccation-sensitive species, P. nubifer, which is not able to accumulate trehalose for entering anhydrobiosis, the levels of both T6P and G6P decreased sensibly, probably reflecting a depression of metabolism leading to death (Fig. S2).

Trehalase inhibition in larvae

Larvae were injected with a trehalase inhibitor, Validoxylamine A (VAA), or with water as a control (cf. methods in the main text). One day after injection and until the induction of anhydrobiosis by desiccation, VAA did not show any toxic effect, compared to control larvae. After rehydration of dry larvae, more than 80% of VAA-injected larvae did not recover correctly and eventually died, whereas more than 90% of the control water-injected larvae recovered normally (Fig. 3C, main text). Trehalase content was estimated in dry larvae and 20 h after rehydration (see methods in the main text). In control dry larvae, the mean trehalose amount was 40.5 µg/larva (i.e an increase of about 13-fold compared to the mean trehalose content in wet larvae as shown in Fig. 3, main text) and this value dropped to 25.3 µg/ larva after 20 h of rehydration, due to activated trehalase activity (Fig. S3). In contrast, VAA-treated larvae accumulated only 25.5 µg/larva (i.e an increase of about 8-fold compared to the mean trehalose content in wet larvae as shown in Fig. 3, main text), but 20 h after rehydration this mean value kept stable (28.5 µg/larva), in agreement with the expected trehalase inhibition. The side effect limiting trehalose accumulation in VAA-treated dry larvae could be due to unspecific interaction of VAA with trehalose synthesis enzymes, such as trehalose-6-phosphate phosphatase that would recognize a structure similar to trehalose (1).
Fig. S3. Effect of trehalase inhibition on trehalose accumulation in larvae. Larvae injected either with water (Control, grey bars with black dots) or with trehalase inhibitor (VAA, white bars with white dots) were subjected to desiccation and subsequent rehydration. The amount of trehalose (µg/larva) was investigated in the anhydrobiotic (Dry) form and 20 h after rehydration (R20h). Bars show the mean values with standard deviation.

Antioxidant potential in larvae

In order to verify the link between trehalose degradation fueling Pentose Phosphate Pathway (PPP) and the recovery of antioxidant potential in rehydrating larvae (see the main text), the total antioxidant potential of larvae was investigated. Compared to wet larvae, the total antioxidant potential of mock-injected control dry larvae increased by about 2-fold and subsequently returned back to the same level as wet larvae 20 h after the rehydration of these control larvae (Fig. S4). In contrast, VAA-injected larvae did not show significant increase of their antioxidant potential in the dry state and kept similar levels 20 h after rehydration, with no significant difference with the antioxidant potential of wet larvae (Fig. S4). In dry larvae, the increase of total antioxidant potential was observed only in control larvae but not in VAA-treated larvae. These samples differ by the higher trehalose accumulation in control larvae (Fig. S3). Thus, trehalose could be directly responsible for the enhancement of the total antioxidant potential. Actually, trehalose was shown to exhibit direct antioxidant function, protecting unsaturated fatty acids from oxidation (2). Concerning the relation between trehalose degradation and the enhancement of antioxidant function, our data could not confirm, neither refute the hypothesis. Due to the negative side effect of VAA on trehalose accumulation during desiccation (Fig. S3), rehydrating larvae could fuel PPP with their residual stock of glucose in a similar way as control larvae, even with inhibited trehalose degradation.
Fig. S4. Effect of trehalase inhibition on the total antioxidant potential of larvae. Larvae injected either with water (Control) or with trehalase inhibitor (VAA) were subjected to desiccation and subsequent rehydration. The total antioxidant potential, expressed as mM ascorbic acid equivalents per larva, was estimated in dry larvae and larvae 20 h after rehydration for both treatment and in control wet larvae as well. Bars represent the mean values with standard deviation. Significant differences are shown (***: p value <0.001; *: P-value <0.05; one-way ANOVA followed by Tukey’s test).

Trehalase knock-down in Pv11 cells

Trehalase gene expression knock-down efficiency was first assessed on Pv11 cells from P. vanderplanki. Compared to Pv11 cells mock-treated or treated with random siRNAs, Pv11 cells treated with specific siRNAs against trehalase sequence showed inhibited trehalase expression by about 85% for TREH-408 and about 80% for TREH-1165 (Fig. S5). The siRNA TREH-1939 did not show significant knock-down of trehalase gene expression (Fig. S5). The most effective siRNA (TREH-408) was used for subsequent experiments.

Next, Pv11 cells were transfected with either control random siRNA (Nc1) or with TREH-408 siRNA. After recovery, cells were pretreated with 600 mM trehalose mixture and then desiccated for 25 days. One hour after rehydration, the cell viability of Nc1 and TREH-408 samples was assessed by trypan blue staining as described in Watanabe et al. 2016 (3) and there was no significant difference between the treatments (Fig. S6A). There was also no significant difference in the mean cell number 1h after rehydration (Fig. S6B). However, 7 days after rehydration, Pv11 cell growth was significantly inhibited in TREH-408 samples, with a final cell number 3.8-fold lower, compared to control Nc1 samples (Fig. S6B). Thus, inhibition of trehalase expression did not affect cell viability just after rehydration, but impaired subsequent cell growth, compared to control. Since Pv11 cells are grown in a sugar-rich medium, the inhibition of intracellular trehalose degradation is not expected to affect glycolysis and energy metabolism in a sensible manner as
in larvae (cf. main text). However, the inhibition of intracellular trehalose degradation by trehalase during the first steps of rehydration is likely to influence cell growth through another metabolic effect, such as early PPP activation enhancing antioxidant potential as hypothesized above. Alternatively, inefficient depletion of intracellular trehalose could directly influence cell growth though a negative effect of trehalose itself on cell proliferation, as shown in mammalian cells (4). In conclusion, although the exact mechanism remains to be determined, it is clear that trehalose degradation after rehydration is essential for the normal recovery of dry cells, independently from the energy metabolism through glycolysis.

Fig. S5. Knock-down of trehalase expression by RNAi.
Relative expression of trehalase gene in Pv11 cells after control mock transfection (CTRL), after transfection with one of the control random siRNAs (Nc1 and Nc2), or transfection with one of the siRNAs against trehalase sequence (TREH-408, TREH-1165 or TREH-1939). The relative expression levels were normalized to Pvef1-alpha expression in each sample. Bars represent the mean of 3 replicates and the error bars show SEM.
Fig. S6. Effect of trehalase RNAi on Pv11 cells desiccation tolerance. Pv11 cells were transfected either with random control siRNA (Nc1) or with trehalase siRNA (TREH-408) and subjected to desiccation and subsequent rehydration. (A) Viability of Pv11 cells estimated 1 hour after rehydration (R1h). (B) Cell growth observed between 1 hour (R1h) and 7 days (R7days) after rehydration. Dots represent individual values for Nc1 and squares represent individual values for TREH-408. Bars represent the mean values with standard deviation. Significant differences are shown (*: P-value <0.05; Multiple t-tests).
Citric Acid Cycle and energy metabolism

The citric acid cycle (CAC) or Krebs cycle is a cyclical series of enzymatic reactions in all aerobic organisms that generate energy in the form of adenosine triphosphate (ATP). During desiccation of *P. vanderplanki* larvae, the content of citrate and cis-aconitate increased gradually and this accumulation was accelerated during the last hours of desiccation, reaching a maximum after 48h of desiccation, in the fully anhydrobiotic state (Fig. S7). In contrast, the down-stream CAC metabolites (2-oxoglutarate, Succinate, Fumarate and malate) showed decreased content during desiccation (Fig. S7). During the first hours following rehydration, the stock of citrate and cis-aconitate was consumed rapidly, allowing a quick restart of energy production in the mitochondria (Fig. S7, Fig. 4, main text). Just after rehydration, the down-stream CAC metabolites levels rose sensibly, but they returned back to levels similar to those observed in the wet larvae only 12-24h after rehydration (Fig. S8), when trehalose degradation ceased fuelling pentose-phosphate pathway and carbohydrates were fully redirected to CAC through glycolysis (see main text).

Fig. S7. Comparative metabolic profiling of *P. vanderplanki* (red circles) and *P. nubifer* (green diamonds) larvae during desiccation: change in abundance of CAC intermediates. Corresponding to changes in metabolite content, Y-axes represent arbitrary units based on the relative metabolite peak area. Standard deviation is shown for D0 and D48 samples obtained in duplicate for *P. vanderplanki.*
Dynamics of amino acids

Fig. S8. Fluctuations in the pool of standard amino acids during the desiccation-rehydration cycle. The green frame marks an increase in content level in response to at least one anhydrobiosis-related stage (desiccation or rehydration) or both. The red frame marks a decrease in content. Standard amino acids without significant changes (p ≥ 0.05) are not shown. The dynamics of each metabolite are shown as dotplots. Time points are indicated by colors (green – D0, red – D48, blue – R3), the averages of three replicates are marked by black dots. Error bars show standard deviation. The y-axes show absolute amounts in pmol per individual. Asterisks indicate significance level (*<0.05, **<0.01, ***<0.001, ****<0.0001).

Across the full list of metabolites identified, 20 proteinogenic amino acids were found. Within the amino acid pool, 13 amino acids showed a significant increase in abundance for at least one of
the investigated stages of anhydrobiosis (alanine, arginine, asparagine, glutamic acid, isoleucine, leucine, histidine, phenylalanine, proline, serine, tryptophan, threonine and valine), three amino acids decreased in level (glutamine, lysine and tyrosine) and six amino acids showed no meaningful shift in content during the desiccation-rehydration cycle (Fig. S8). The biggest increases were noted for threonine (4.2x ↑), proline (3.4x ↑) and serine (2.5x ↑); the largest declines for lysine (5x ↓) and tyrosine (2x ↓). It is unclear whether such changes are due to the degradation of intermediates or whether they reflect other effects of anhydrobiosis on cell physiology. For example, it has been suggested that proline plays an important role in promoting biotic/abiotic stress resistance and lifespan extension, as it has osmoprotective, chelation, signaling and redox homeostasis properties (5, 6, 7). Likewise, the accumulation of threonine is thought to enhance resistance to saline, drought and cold stress in plants (8).

There is a correlation between high levels of alanine and tolerance to freezing in the Antarctic midge B. antarctica (9), as well as with cold hardiness in larvae of the Drosophilid fly, Chymomyza costata (10), and other cold-resistant insects (11, 12). One explanation for this is that alanine potentially increases the overall osmolality of hemolymph as it has colligative properties similar to those of glycerol (13). However, alanine, as a direct product of pyruvate that is less toxic than lactate, may simply represent a benign repository for carbon flowing through the glycolytic pathway under conditions where the CAC is suppressed (9, 14). In P. vanderplanki larvae, the level of alanine did not change significantly during desiccation, but strongly increased on rehydration. Apparently, survival of anhydrobiosis does not require accumulation of alanine beforehand, in contrast to freezing, but alanine production may be essential in rehydration, when larval metabolism starts to recover.
Uric acid degradation pathway

How insects excrete an excess of nitrogenous compounds varies in different taxa and may depend on stage of life cycle or, in some cases, nutritional characteristics (15). Aquatic insects can dispose of toxic ammonia directly, but for terrestrial species it is more common to convert nitrogen to uric acid to conserve water. As for other excreted nitrogenous waste, insects can produce allantoin, allantoic acid, hypoxanthine, xanthine and some amino acids in different species-specific amounts and proportions. With some exceptions, these products constitute a minor component of feces. Urea may also occur in feces, but only in a few insect species is it a major excretory compound (16). At some stages of development or when excretion is impeded, nitrogenous compounds can be stored mainly in the form of non-toxic uric acid (17).

In the metabolome of *P. vanderplanki*, uric acid, allantoin, allantoic acid, and polyamines such as ornithine and putrescine are all among the intermediates whose abundance was most increased during anhydrobiosis (Table S1), with allantoin the most highly represented. Notably, neither allantoin nor allantoic acid were detected in the metabolome of *P. nubifer* larvae (Fig. S9).

**Fig. S9.** Comparative metabolic profiling of uric acid degradation pathway in *P. vanderplanki* (red circles) and *P. nubifer* (green diamonds) larvae. Corresponding to changes in metabolite content,
Y-axes represent arbitrary units based on the relative metabolite peak area. Standard deviation is shown for D0 and D48 samples obtained in duplicate for *P. vanderplanki*.

 Interestingly, allantoin is a metabolite known for its protective and potentially antioxidant function under stress conditions (18, 19). Polyamines, also, are known for their role in enhancing plant tolerance to abiotic stresses, including dehydration and oxidative stress (20). The polyamines ornithine and putrescine showed increasing levels after 3h and 12h of rehydration, respectively, and their presence may attenuate rehydration stress as allantoin, before effective excretion allows the purge of more toxic end products, such as urea (Table S2, Fig. S9).

**Compensation of glutathione system with a new putative antioxidant?**

As a major scavenger of reactive species, glutathione (GSH) can eliminate radicals non-enzymatically by direct interaction with ROS or reactive nitrogen species (21). Oxidative stress leads to an increased consumption of GSH, which under normal conditions should stimulate GSH biosynthesis. Although we expected to find an increase in levels of GSH precursors and the respective biosynthetic enzymes, metabolic profiling of *P. vanderplanki* did not detect any marked shift in GSH content (Fig. 6B, main text). Alternatively, the enzymes involved in GSH biosynthesis can also promote the synthesis of ophthalmic acid (OA) from the precursors 2-aminobutyric acid and γ-Glu-2AB and these compounds were accumulated in desiccated larvae and during the first hours after rehydration (Fig. S10). OA itself was reported to exhibit antioxidant properties (22) and its presence from dry larvae to 3h after rehydration is likely to compensate oxidative stress at these delicate stages, thus minimizing the need for GSH as an antioxidant. In addition, desiccation-inducible isoforms of glutathione peroxidase have been suggested to utilize thioredoxins as a substrate, instead of GSH (23). This specific pathway probably also contributes to the maintenance of GSH homeostasis during anhydrobiosis.

![Fig. S10](image-url). Comparative metabolic profiling of ophthalmic acid pathway in *P. vanderplanki* (red circles) and *P. nubifer* (green diamonds) larvae. Corresponding to changes in metabolite content, Y-axes represent arbitrary units based on the relative metabolite peak area. Standard deviation is shown for D0 and D48 samples obtained in duplicate for *P. vanderplanki*. 


Detoxification of tryptophan degradation products

The kynurenine pathway is the main branch route for tryptophan catabolism and has attracted attention because of its role in a huge range of neurodegenerative diseases in mammals (24, 25, 26). In insects, an impact of intermediates of the kynurenine pathway is not clear beyond their role as precursors for the eye pigment, ommochrome (27, 28). The breakdown of tryptophan via kynurenines (KYN) produces metabolites such as kynurenic acid (KA) and 3-hydroxykynurenine (3-HK), which is highly reactive and produces free radicals (29, 30). Actually, the level of KYN increased in anhydrobiotic *P. vanderplanki* larvae and this high level was maintained during the first hours following rehydration (Fig 6C, main text). Similarly, KA level was higher in dry larvae and decreased gradually after rehydration (Fig. S11). However, despite the highly toxic nature of 3-HK, hydroxylation of KYN to 3-HK has been suggested as the dominant route for tryptophan degradation in insects, implying that the 3-HK level must be efficiently regulated in cells (31).

Genome and transcriptome analysis of *P. vanderplanki* larvae showed that this insect acquired specific enzymes (3-hydroxykynurenine transaminases) able to convert 3-HK to xanthurenic acid (XA) (Fig. 6C, main text). Actually, XA content was increased gradually during desiccation and kept relatively high levels after rehydration (Fig. S11). Therefore, we suggest that XA constitute a safe end product of tryptophan degradation through KYN pathway, minimizing the production of the highly toxic intermediate compound 3-HK and thus enhancing survival of anhydrobiotic larvae after rehydration.

**Fig. S11.** Comparative metabolic profiling of kynurenic acid and xanthurenic acid in *P. vanderplanki* (red circles) and *P. nubifer* (green diamonds) larvae. Corresponding to changes in metabolite content, Y-axes represent arbitrary units based on the relative metabolite peak area. Standard deviation is shown for D0 and D48 samples obtained in duplicate for *P. vanderplanki*.
Material and methods.

Insect rearing and sample preparation for metabolic profiling

P. vanderplanki and P. nubifer larvae were reared on a 1% agar diet, containing 2% commercial milk, under controlled conditions (13 h light: 11 h dark; temperature 27-28°C) according to the established protocol (32): larvae were placed on filter paper (Filter paper qualitative 2, diameter 55 mm, Advantec, Japan) with 0.44 ml of distilled water in a glass Petri dish (diameter 65 mm, height 20 mm), which was set in a desiccator (25 x 25 x 30 cm) with 1 kg of silica gel. For comparative metabolic analysis between anhydrobiotic and non-anhydrobiotic chironomids, 50 larvae were used per treatment. Metabolic profiling included several points (treatments) corresponding to the time in hours from the beginning of desiccation process: 0 h (D0, control); 12 h (D12); 24 h (D24); 36 h (D36); 48 h (D48). D0 and D48 samples were obtained in duplicate. For the non-anhydrobiotic P. nubifer chironomid, sample preparation was stopped after 24 hours of desiccation, because of subsequent larvae's mortality. For anhydrobiotic P. vanderplanki larvae, sample preparation was continued with rehydration course: 1 h (R1), 3 h (R3), 12 h (R12) and 24 h (R24) after adding water.

Measurement of metabolites
Measurement of metabolites was carried out by Human Metabolome Technologies, Inc. (Tsuruoka, Yamagata, Japan). Larvae samples (50 individuals each) were homogenized into 500 µl of methanol containing internal standards and then, after mixing with 500 µl chloroform and 200 µl water, samples were centrifuged at 2,300 g for 5 min. The aqueous supernatant fraction was filtered with 5 kDa exclusion filter tubes (UltraFree MC PLHCC HMT, Millipore) and then dried-up. Samples were recovered into 50 µl MilliQ water, prior to metabolite analysis using the capillary electrophoresis time of flight-mass spectrometry: Agilent CE-TOFMS system (Agilent Technologies, Santa Clara, CA, USA) in two modes for cationic and anionic metabolites. All metabolites were analyzed using fused silica capillary (50 µm i.d.; 80 cm total length). In the anionic analysis, the sample was injected at a pressure of 50 mbar for 25 s; the applied voltage was set at 30 kV. The cationic metabolites samples were injected at a pressure of 50 mbar for 10 s; the applied voltage was set at 27 kV. Both spectrometers were scanned from mass-to-charge (m/z) 50 to 1000. Peaks detected in CE-TOFMS analysis were extracted using automatic integration software MasterHands ver. 2.9.0.9 (Keio University, Japan). The peaks were annotated with the 272 specified putative metabolites from package in the HMT metabolite database (Human Metabolome Technologies, Inc., Tsuruoka, Yamagata, Japan) on the basis of their MT in CE and m/z values determined by TOFMS. The tolerance range for the peak annotation was configured at 0.5 min for MT and 50 parts per million for m/z. The identification and quantification of each metabolite was performed according to a previously reported method (34).

Metabolome data analysis
Analysis of metabolic profiling data was performed by Metabo Analyst 4.0 R package (http://www.metaboanalyst.ca/). The dataset of detected compounds was filtered to remove metabolites with 2/3 of missing values. To replace missing concentration values of low abundance metabolites (below detection threshold), marked as “ND”, we used half of the minimum positive values in the original data. The normalization procedure and statistical analysis for triplicate samples (D0, D48 and R3) are described in the main text.

For reconstruction of metabolic pathways and integration with transcriptomics data we applied KEGG (Kyoto Encyclopedia of Genes and Genomes) PATHWAY Database on available species of Diptera and BLASTp search. As a model for comparison, we used a congeneric species from the same genus, Polypedilum nubifer, which is sensitive to desiccation. It is, at one hand, a good example for comparative metabolomics studies, but, on another hand, there is no way to conduct full desiccation-rehydration cycle in desiccation-sensitive chironomid. We found, however, significant differences between these two species even in early stages of desiccation. The most representative cases were included to this study.
Determination of total antioxidant potential
Larvae treated with either the trehalose inhibitor Validoxylamine A (VAA) or water (as control) were desiccated and rehydrated following the protocol described in the main methods part. The total antioxidant capacity of wet control larvae, of VAA- and water-injected larvae in the dry state and of VAA- and water-injected larva collected 20h after rehydration was determined with the Total Antioxidant Capacity Assay kit (Metallogenics, Chiba, Japan), according to the instructions of the manufacturer. The principle of this assay is based on the fact that antioxidants in the sample can reduce Cu^{2+} to Cu^{+}. Cu^{+} is chelated by bathocuproine and yields orange-colored complex that is detected. Thus, this method allows to detect all antioxidants in the samples, proteins and small molecules as well. Briefly, larvae were individually homogenized in 10µl PBS and homogenates were centrifuged at 13,500 RPM for 6s, before collecting 5µl of the supernatant for immediate reaction with chelate solution, colorimetric solution and stop solution, successively. The OD at 492 nm was determined before and after the reaction with cupric ion colorimetric solution on a 96-wells plate, with an E-Max Plus microplate reader (Molecular Devices, San Jose, CA, USA), associated with SoftMax Pro 6.4 software (Molecular Devices, San Jose, CA, USA). The total antioxidant potential index was calculated in reference to the antioxidant potential of an ascorbic acid standard, according to the manufacturer's instruction.

Trehalase gene knock-down in Pv11 cells
Knock-down of trehalase gene was performed by RNAi, according to the method described previously (35, 36), with minor modifications. PvTrehalase gene siRNAs were designed using siDirect (37). The uniqueness of each candidate siRNA was confirmed by BLAST search of the P. vanderplanki genome (Midgebase: bertone.nises-f.affrc.go.jp/midgebase) to minimize off-target effects. All designed siRNAs were synthesized and annealed by Hokkaido System Science Company. The sequences of siRNAs were as follows: TCREH-408sens: 5’-GACUUUGGUUGAAUGAAATT-3’; TCREH-1165sens: 5’-GAACAUUAUGCAAAUUAATT-3’; and TCREH-1939sens: 5’-CCAUAACAUAGGAAAATT-3’. In addition, two types of siRNA with randomized sequences were designed as negative controls. BLAST searches showed that the sequences were not present in the P. vanderplanki genome: NC1 sens: 5’-GCACUGCUACGAUCG-UUAATT-3’; and NC2 sens: 5’-GUAGAGAGCCGCAUCUAAATT-3’. Transfection was performed as follows: samples of 10⁶ Pv11 cells were transfected with either siRNAs (0.5 µM final concentration) or TE (10 mM Tris-HCl, pH 8, 1 mM EDTA) buffer only as a control mock treatment using the 4D-Nucleofector System (Lonza) according to the manufacturer's instructions with a DC134 pulse code in SG medium. Following transfection, Pv11 cells were grown in 1 mL of complete IPL-41 medium in a single well of a 12-well plate for 48 h and subsequently incubated for an additional 48h in 600 mM trehalose preconditioning mixture. After incubation, Pv11 cells were collected by centrifugation at 500 x g for 5 min and total RNA was extracted with the ReliaPrep RNA Tissue Miniprep System (Promega), followed by reverse-transcription with a Ready-To-Go T-primed First-Strand cDNA Synthesis Kit (GE Healthcare) before real-time PCR analysis. Alternatively, after 600 mM trehalose preconditioning mixture treatment, Pv11 cells were desiccated following the protocol described previously (Watanabe et al., 2016), with slight modifications. The equivalent of 10⁶ cells suspended into a 10 µl droplet of 600 mM trehalose mixture was placed at the center of a tissue culture dish (DB Falcon, 353001) and 3 additional droplets of 600 mM trehalose mixture only were placed in the vicinity in order to respect the cell concentration and solute quantity for the desiccation treatment, which was performed in a desiccator as described in Watanabe et al. (2016) for the rest of the procedure. Cell viability and cell number were then assessed 1h and 7 days after rehydration, as described before (3).

Real-time quantitative PCR
The real-time quantitative PCR primers were designed using Primer3 Plus software: Pv Treh-RTP-F1: 5’-AACTTATGTTGAAACCACAAATGA-3’; Treh-RTP-R1: 5’-TTCTGCAATAATGTTCTTTTGCTT-3’; PvEf1a-RTP-F1: 5’-AACTGACAAACCATTGCG-3’; PvEf1a-RTP-R1: 5’-AACTGACAAACCATTGCG-3’. The relative expression of Trehalase gene was quantified using the CFX96 RT-PCR detection system (Bio-Rad) with an SYBR Premix Ex
Taq kit (Tli RNase H Plus; TaKaRa Bio). PCR was performed using two-step cycling conditions: 95°C for 60 s; 95°C for 10 s and 60°C for 40 s, repeated for 40 cycles; 95 °C for 5 s. Melt curve analysis of qPCR samples revealed that there was only one product for each gene primer reaction. Results were normalized to *PvEf1-alpha* (GenBank accession number AB490338) as an internal control. Relative expression data were obtained in triplicate.
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