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RESEARCH ARTICLE

Metabolome dynamics of diapause in the butterfly *Pieris napi*: distinguishing maintenance, termination and post-diapause phases

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ABSTRACT

Diapause is a deep resting stage facilitating temporal avoidance of unfavourable environmental conditions, and is used by many insects to adapt their life cycle to seasonal variation. Although considerable work has been invested in trying to understand each of the major diapause stages (induction, maintenance and termination), we know very little about the transitions between stages, especially diapause termination. Understanding diapause termination is crucial for modelling and predicting spring emergence and winter physiology of insects, including many pest insects. In order to gain these insights, we investigated metabolome dynamics across diapause development in pupae of the butterfly *Pieris napi*, which exhibits adaptive latitudinal variation in the length of endogenous diapause that is uniquely well characterized. By employing a time-series experiment, we show that the whole-body metabolome is highly dynamic throughout diapause and differs between pupae kept at a diapause-terminating (low) temperature and those kept at a diapause-maintaining (high) temperature. We show major physiological transitions through diapause, separate temperature-dependent from temperature-independent processes and identify significant patterns of metabolite accumulation and degradation. Together, the data show that although the general diapause phenotype (suppressed metabolism, increased cold tolerance) is established in a temperature-independent fashion, diapause termination is temperature dependent and requires a cold signal. This revealed several metabolites that are only accumulated under diapause-terminating conditions and degraded in a temperature-unrelated fashion during diapause termination. In conclusion, our findings indicate that some metabolites, in addition to functioning as cryoprotectants, for example, are candidates for having regulatory roles as metabolic clocks or time-keepers during diapause.

KEY WORDS: Hypometabolism, Stress, Cryoprotectant, Developmental plasticity, Biological clock, Phenology

INTRODUCTION

Life in seasonal environments requires synchronizing the life cycle to periods of resource availability (Nelson et al., 2010). Diapause is a deep resting stage that allows insects to temporally avoid stressful periods of resource limitation that is generally characterized by reproductive and/or developmental arrest, as well as deep metabolic suppression (Tauber et al., 1986). Because the decision to enter diapause is often facultative, with individuals deciding to either develop/reproduce directly or interrupt development/reproduction and enter diapause, it exemplifies adaptive developmental plasticity (Gotthard and Nylin, 1995). Although diapause induction has been studied extensively during the last 100 years on many functional levels (Tauber et al., 1986; Nelson et al., 2010; Lehmann et al., 2015; Pruisscher et al., 2017), the physiological mechanisms of diapause termination remain poorly understood (Hodek, 2002; Hodek, 1996).

Studying the transition from diapause maintenance through termination to post-diapause quiescence is challenging for several reasons. First, the phenotype often remains relatively unchanged after diapause termination (Hodek, 1996; Hodek, 2002), making it difficult to identify the pre- and post-termination states for analysis. Second, clear molecular or physiological markers of diapause termination that could be used for such research design are lacking (Yocum et al., 2011). Third, while the proximate, downstream mechanisms likely involve hormonal drivers, such as diapause hormone and ecdysone (Zhang et al., 2004; Jiang et al., 2014; Denlinger et al., 2012), the upstream, primary factors of termination remain unknown. Finally, in many insects chilling is needed for diapause to progress to termination, but the molecular mechanisms of this process are completely unknown. This is partly due to temperature having multiple roles in diapause, as a direct stressor (Danks, 2007), a modulator of metabolism and energy expenditure (Hahn and Denlinger, 2007; Williams et al., 2016), and a pacemaker of time-keeping mechanisms (Stålhandske et al., 2015; Posledovich et al., 2015), further complicating the identification of good candidates for the primary factors of termination.

Although the mechanisms behind the termination of endogenous diapause generally are poorly known, the sugar-induced developmental block of diapause in *Bombyx mori* deserves special mention. The embryonal diapause of *B. mori* is maternally induced and the diapause phenotype of the eggs is rapidly established after oviposition. This phenotype includes the accumulation of sorbitol and glycerol in large amounts (Chino, 1958). In a series of experiments, researchers confirmed the primary role of sorbitol to be in developmentally arresting the embryo in the G_2 phase of the cell cycle (Kiguchi et al., 1990; Horie et al., 2000; Moribe et al., 2001). Termination of diapause is then dependent upon the degradation of sorbitol (Horie et al., 2000), suggesting that metabolites might have regulatory roles during diapause timing and termination (Xu et al., 2012).

Here, in order to gain detailed insights into roles of metabolites during diapause termination, we characterized metabolome dynamics

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during diapause in the butterfly *Pieris napi* (Linnaeaus 1758) (Lepidoptera: Pieridae) with a multi-layered clustering analysis combined with a high-resolution temporal design and temperature manipulation. *Pieris napi* has a facultative pupal diapause with a clear diapause phenotype, and the temperature-dependent dynamism of its diapause termination has been described (Posledovich et al., 2015; Lehmann et al., 2016, 2017a). Although it is expected that metabolome dynamics should be tightly linked to the development of cold tolerance (one important aspect of diapause), our main interest is to study metabolite dynamics around diapause termination in order to gain mechanistic insights into this important biological timing event. Because there are examples of developmental blocks during diapause that are maintained by metabolites, such as sorbitol in *B. mori* (Horie et al., 2000), metabolome dynamics might reveal transitions through diapause and pinpoint key shifts related to diapause termination in *P. napi*.

Although the induction of diapause in *P. napi* is primarily driven by photoperiod (Friberg et al., 2011; Lehmann et al., 2016), its termination is driven by temperature (Posledovich et al., 2015; Lehmann et al., 2016, 2017). Pupae kept at warm temperatures (≥15°C) will remain in diapause maintenance, unable to terminate diapause, while pupae moved to cold temperatures (≤10°C) will terminate diapause (a process requiring approximately 3 months in the studied population; see inset in Fig. 1A). As diapause can be induced quite early during summer, the low-temperature requirement is likely a fail-safe mechanism that prevents diapause termination during late summer or early autumn. By studying pupae kept at high or low temperature during diapause in a time-series experiment (Ragland and Keep, 2017), we can contrast cold tolerance and metabolome dynamics associated with diapause maintenance and diapause termination (Fig. 1A). The overall goal of this study is to investigate the extent to which diapause maintenance and termination pupae differ in key physiological traits. More specifically, our design allows us to test whether (1) low temperatures are needed for the development of cold tolerance, (2) diapause at high or low temperature is associated with differences in energy metabolism and (3) diapause termination at low temperature is associated with a reorganization in the metabolome. Of particular interest is the identification of potential metabolites that only are accumulated or degraded in the group, based on previous studies (Posledovich et al., 2015; Lehmann et al., 2017a), that we know is terminating diapause.

MATERIALS AND METHODS

Animal rearing

Experiments were performed between November 2014 and December 2015 at the Department of Zoology at Stockholm University, Sweden. *Pieris napi* eggs were collected from wild plants from two sites (~20 km apart) in Skåne, southern Sweden (Kullaberg: 56°18′N, 12°27′E; and Vejbystrand: 56°18′N, 12°46′E), and brought to the laboratory during the summer/autumn in 2013 and 2014, and reared as described previously (Lehmann et al., 2016). For cold-tolerance assays, F2 larvae were mass-reared in groups of 100 in containers (0.6×0.4×0.4 m) and kept in a temperature-controlled room, and for the metabolome assay larvae were reared in groups of five in smaller containers that were kept in a climate cabinet (KB8400L, Termaks, Bergen, Norway). In both the room and the climate cabinet, four conditions were used. (1) Directly developing larvae and pupae were reared under long day conditions (22 h:2 h light:dark, 20°C). (2) Diapause was induced by rearing larvae under short day conditions (10 h:14 h light:dark, 20°C). Pupae were kept for 10 days at 20°C and subsequently divided into one of two treatments. (3) To stimulate diapause termination, one group of pupae were moved to 10°C for 7 days and finally to 2°C, which was the holding temperature throughout diapause, which lasted 5 months. After 5 months at 2°C, pupae were moved to 10°C for 7 days, and finally to 20°C until they hatched. (4) To maintain diapause maintenance, a fourth group of pupae was left at 20°C and not moved to lower temperatures when 10 days old. This setup allows us to partition out processes related to diapause termination from processes related to diapause maintenance (Fig. 1A, Table S1).

Cold tolerance

Cold tolerance of larvae and pupae undergoing direct development, diapause maintenance or termination was investigated through assessment of supercooling point (SCP) and cold shock tolerance. The cold shock conditions reflect a cold night in natural overwintering habitats. Not all cold tolerance metrics were taken from all groups (Table S1). We investigated how cold tolerance is developed early in diapause, and dismantled upon resumption of development after winter. Because cold shocks can carry latent effects, survival after the chronic cold exposure and cold shock treatments was assessed as successful post-diapause hatching of adults.

The SCP reflects the freezing point of a liquid cooled below 0°C and is measured as the freezing exotherm released from a sample when internal water shifts state from liquid to frozen (Sinclair et al., 2015). The freezing exotherm (SCP) was measured with K type thermocouples connected to a TC-08 Picologger (Pico Technologies, Eaton Socon, UK) using a sampling rate of 1 Hz. Individual pupae were weighed to the closest 0.1 mg on an analytical balance (Sauter RE1614, Ballingen, Germany) and placed in 15 ml plastic tubes. The tubes were submerged in a programmable water bath (FP40+HL, Julabo, Seelbach, Germany) programmed to go from 10 to −30°C at a rate of 0.02°C min^{-1}. The thermocouple was not fastened to the cuticula as preliminary experiments showed clear exotherms also without thermal paste or adhesive putty.

For cold-shock tolerance trials, pupae were weighed to the nearest 0.1 mg on an analytical balance (Sauter RE1614) and placed individually in 1.5 ml microtubes, after which they were submerged in groups of 24 (12 females and 12 males) in a programmable water bath (FP40+HL, Julabo) for 20 h using a cooling and heating rate of 0.02°C min^{-1}. Four temperatures were used: −5, −10, −15 and −20°C. Survival was assessed as the capacity to survive to normal adult hatching after diapause. This was within 2 weeks of the shock for directly developing pupae, and after a 5 month winter for the diapause termination pupae. For diapause maintenance pupae, survival was assessed 24 h after the treatment (Lehmann et al., 2016).

Metabolomics

Whole pupae were homogenized twice in 400 µl of 70% ethanol and the extracts were subjected to a complex analysis of major metabolites using a combination of mass spectrometry-based analytical methods as described earlier (Koštál et al. 2011, 2016). Briefly, low-molecular-weight sugars and polyols were determined after o-methyloxime trimethylsilyl derivatization using a gas chromatograph (GC) with a flame ionization detector (GC-FID-2014) equipped with an AOC-20i autosampler (both from Shimadzu Corporation, Kyoto, Japan). Profiling of acidic metabolites was done after treatment with ethyl chloroformate under pyridine catalysis and

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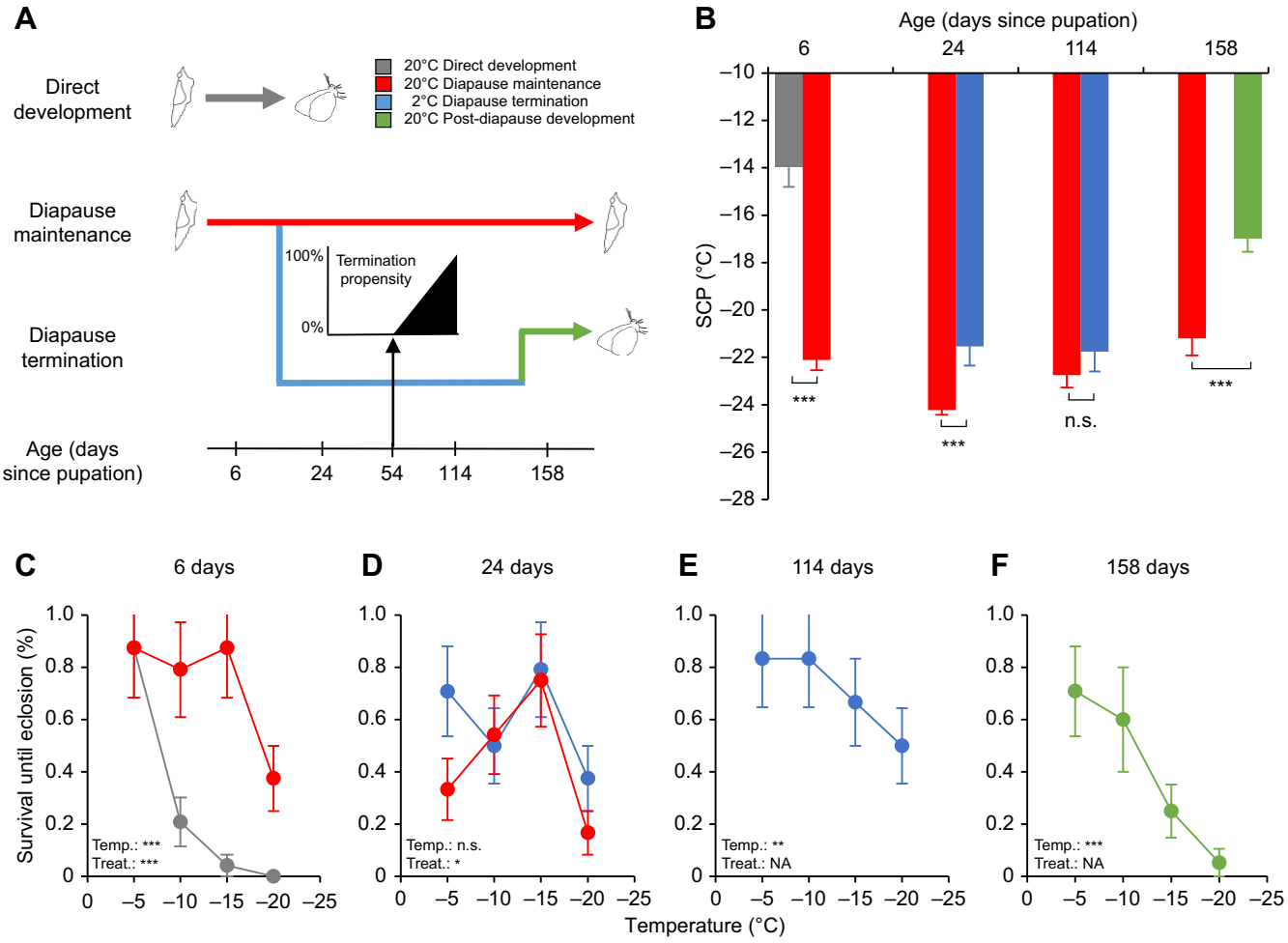

Fig. 1. Cold tolerance of *Pieris napi* during different phases of direct (grey) or diapause maintenance (red), diapause termination (blue) or post-diapause development (green). (A) Schematic overview of the experimental design showing the four main groups. The treatments share a timeline. The inset shows diapause termination propensity in the studied population (Lehmann et al., 2017). (B) Supercooling points (SCP) of pupae from the three treatments. Treatments were compared within time points with a binomial generalized linear model (***P<0.001; n.s., P>0.05). The full test can be seen in Table S2. (C–F) Survival after a 20 h cold shock. Note that both diapause and direct pupae in C are at 20°C, while D shows diapause termination pupae moved to 2°C compared against diapause-maintenance pupae left at 20°C. For statistical analyses, Temp.=temperature effect and Treat. =treatment group effect. NA, not tested; n.s., P>0.05; *P<0.05; **P<0.01; and ***P<0.001. Full tests can be seen in Table S3. Data are means±s.e.; in C–F, s.e. is calculated as square root (pq/n), where pq is the mean proportion and n is the group sample size. In B, N=16 per point and in C–F, N=24 per point.

simultaneous extraction in chloroform (Hušek and Šimek, 2001). We used a Trace 1300 GC combined with single quadrupole mass spectrometry (ISQ-MS) (both from Thermo Fisher Scientific, San Jose, CA, USA) and a liquid chromatograph Accela LTQ XL with a linear ion trap combined with the high-resolution mass spectrometers Q Exactive Plus coupled with Dionex Ultimate 3000 (all from Thermo Fisher Scientific). The metabolites were identified against relevant standards and subjected to quantitative analysis using an internal standard calibration method. All standards used were purchased from Sigma-Aldrich (Saint Louis, MO, USA).

Statistical analysis

Generalized linear models (GLM) were employed for analyses of cold tolerance. In the text below, mass is fresh mass in grams, age is days since pupation, and treatment group is direct, diapause termination or maintenance, unless stated otherwise. In all tests a fully factorial design was used. Nonsignificant interactions and main effects were removed from final models (Sokal and Rohlf, 2003) and model improvement was tracked through the Akaike information criterion (AIC). In case of significant effects, *post hoc* comparisons of main level effects were performed with Bonferroni multiple-group corrections. All statistical tests except the cluster analyses were performed with IBM SPSS statistics 23.0 (IBM SPSS Inc., Chicago, IL, USA).

The SCP was analyzed on the data split by age with a GLM employing a normal distribution, with SCP as the dependent variable, mass as the covariate, and path and sex as factorial explanatory variables. Sex was removed during model selection. Cold shock tolerance was analyzed with a GLM employing a binomial distribution, with survival until adult eclosion as the dependent variable, mass as the covariate, and path, sex and age as factorial explanatory variables. Sex was removed during model selection. Because the interaction between age and path was significant, the models were rerun on the data split by age. Relative content of total sugars and amino acids (µg mg^{-1} fresh mass, FM) were added as dependent variables in separate GLMs employing a normal distribution with path, age and sex as factorial explanatory variables. Sex was removed during model selection. Because the

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interaction between age and path was significant, the models were rerun on the data split by age. Differences in summed metabolite amounts over the diapause period (24 to 144 days) were analyzed with a GLM using a normal distribution with path and sex as explanatory variables. Sex was removed during model selection.

To visualize overall metabolome dynamics, we first employed principal component analyses (PCA) on the entire dataset. The metabolite data were then standardized (mean zero and unit variance) per metabolite across all time points in the diapause maintenance and termination groups (referred to as treatment group in this analysis). Clustering of metabolite profiles (mean standardized concentrations per time point and treatment group) was performed using DBSCAN (density-based spatial clustering of applications with noise) (reachability distance=0.75, reachability minimum number of points=4) (Ester et al., 1996). DBSCAN leaves metabolites that do not fit into any high-density region unassigned. For visualization purposes, hierarchical clustering of the DBSCAN clusters (average cluster profiles) was performed for the diapause termination group and used to order the heatmap rows. Differences across treatment groups in mean metabolite amounts between time points 24 and 114 days (in $\log_2$ fold change of nmol mg^{-1}) were tested with a Mann–Whitney *U*-test (adj. $P<0.05$). A two-way factorial ANOVA with treatment group and time as categorical explanatory variables was used to compare profiles between the treatment groups by testing for significant interactions between time and treatment group. These analyses were performed in R (https://www.r-project.org/). Scripts and workflow description can be found at: https://bitbucket.org/scilifelab-lts/c_wheat_1606/get/paper_version.zip.

RESULTS

Diapause, but not temperature, had large and rapid effects on cold tolerance

To test whether cold tolerance develops as part of the diapause phenotype and its temperature dependence, we compared SCP (the point of internal ice formation) and cold shock tolerance among the treatment groups, to separate out the effects of diapause from the commonly confounding effects of lower temperature (Fig. 1A). Already at 6 days after pupation, diapausing pupae had lower SCP and better cold shock tolerance than direct developing pupae (Fig. 1B,C, Tables S2 and S3). Transfer to the diapause-holding temperature of 2°C (i.e. diapause termination group) did not significantly lower SCP; instead, SCP significantly increased over the diapause maintenance group at 24 days (Fig. 1B). The transfer to 2°C had a marginal effect on cold shock tolerance, which at 24 days was higher than in the diapause-maintenance pupae left at 20°C (Fig. 1D). This effect is driven by better survival at the mildest tested temperature (−5°C). At 114 days, when diapause-termination pupae transitioned to post-diapause quiescence, SCP was as high as in maintenance pupae left at 20°C (Fig. 1B). At day 144, pupae were moved to 10°C for 7 days and then to 20°C. This increase in temperature had a major impact on both SCP and cold shock tolerance in pupae tested at day 158. In this age group, SCP had increased to values similar to those of direct developing pupae (Fig. 1B), while cold shock tolerance had decreased, albeit not to the same level of direct developing pupae (Fig. 1C,F).

Content of total sugars, but not amino acids, was strongly associated with the decision to enter diapause

Already after 3 days (i.e. before being moved to 2°C) diapause pupae had significantly higher sugar and amino acid content than direct developing pupae (Fig. 2, Tables S4 and S5). The content

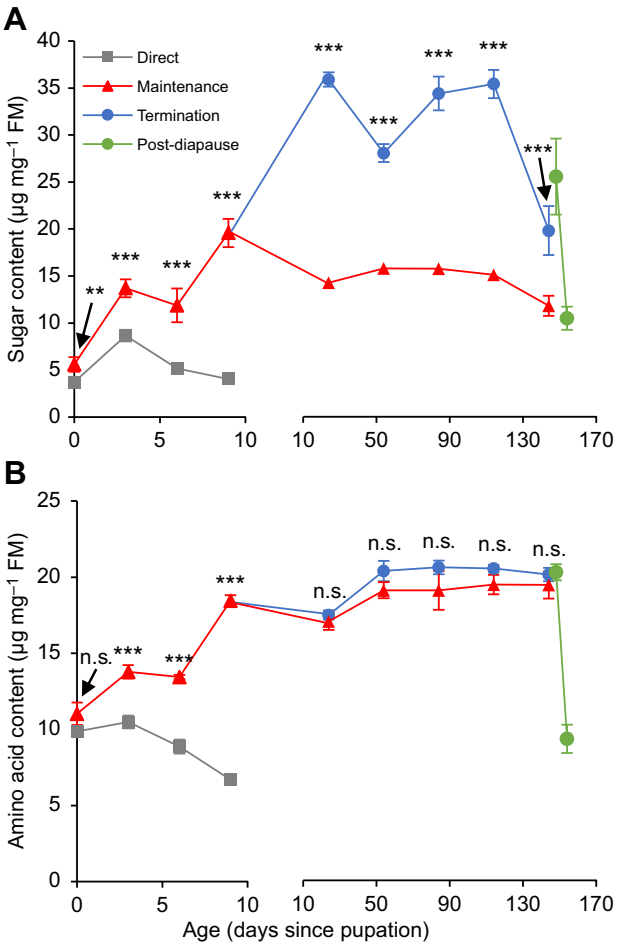

Fig. 2. Total sugar and amino acid content in *Pieris napi* undergoing direct or diapause development. (A) Total sugar; (B) total free amino acids. Grey, red, blue and green symbols indicate direct pupae, and diapause pupae at 20°C (maintenance), 2°C (termination) and at 20°C after removal from cold (post-termination), respectively. Each point is the mean±s.e. of six animals (3 males and 3 females, sexes are pooled). Asterisks denote significant differences between treatment group within an age group (n.s., $P>0.05$; **$P<0.01$; ***$P<0.001$). Note that for ages 0–9, direct pupae were compared against diapause maintenance pupae, and for 24–144, diapause maintenance pupae were compared against diapause termination pupae. For post-diapause 148 and 154, no comparison was possible.

increased steadily throughout the diapause initiation phase, then remained relatively stable during diapause maintenance. Interestingly, even though cold shock tolerance did not differ between diapause-maintenance or -termination pupae at 24 days (Fig. 1A,D), total sugar content was significantly higher in the latter group (Fig. 2A). A similar effect was not seen for total amino acids, which were accumulated at equal total amounts in both diapause groups (Fig. 2B). After diapause was terminated and pupae were moved to high temperature, both sugar and amino acid content rapidly decreased.

Overall metabolome dynamics was shaped by developmental path

A PCA was conducted to assess the main factors grouping our time-series data on the metabolome. The first principal component (PC1), which accounted for 31% of total variation, represents a diapause progression axis where direct pupae and early diapause pupae are clustered at one end (negative PC1), while diapause-

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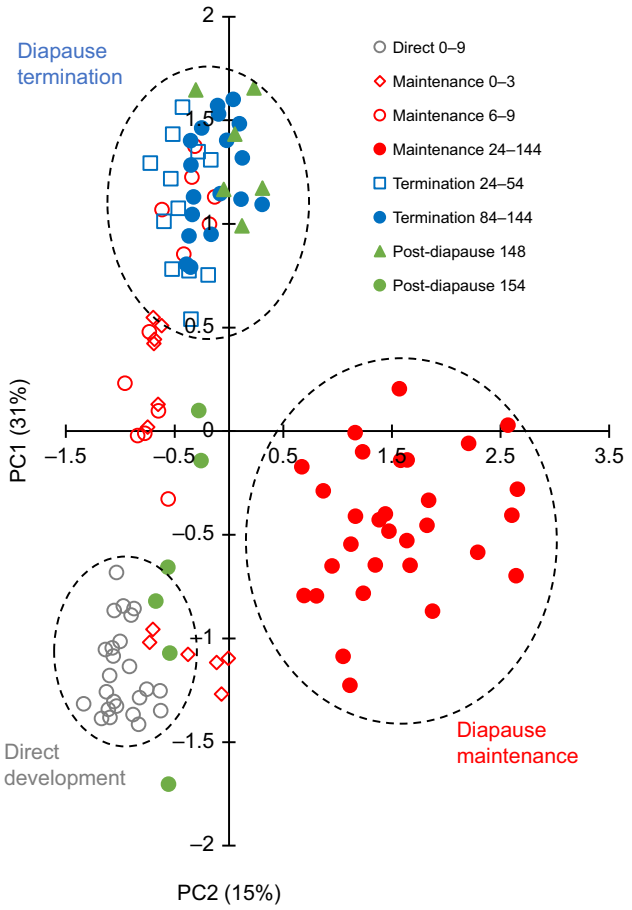

Fig. 3. Overall metabolomics of direct and diapause development in *Pieris napi*. Main explanatory axes of a principle component analysis (PCA) of pupae undergoing direct or diapause development. Diapause 25–54 represents pre-termination diapause (endogenous diapause) while 84–144 represents post-termination diapause (exogenous diapause or post-diapause quiescence). Grey, red, blue and green symbols indicate direct pupae, and diapause pupae at 20°C (maintenance), 2°C (termination) and at 20°C after removal from cold (post-termination), respectively. Loading values and further analyses can be found in Fig. S1.

termination pupae are clustered at the other end, with diapause-maintenance pupae scattered in between (Fig. 3). Thus, without any cold signal, diapause initially increases along the PC1 axis (maintenance 0–9, Fig. 3), followed by a shift along the PC2 axis, accounting for 15% of total variation, that is never seen in the termination group (maintenance 24–144, Fig. 3). In contrast, diapause termination only shifts to more extreme values on the PC1 axis, followed by a return to the direct developing cluster after the transfer to high temperature (post-diapause 148–154). Loading values show that the majority of metabolites accumulated in pupae undergoing diapause, regardless of temperature (Fig. S1). Only a few metabolites were accumulated in higher amounts in direct developing pupae compared with pupae in diapause, namely, aspartate, adipic acid, ribitol and glucose. However, these were accumulated at very low total amounts and direct developing pupae are not discussed further.

Metabolite dynamics during diapause termination was shaped by temperature

In order to understand how metabolite profiles are shaped by temperature and diapause progression, we performed a clustering analysis using DBSCAN, which allowed us to identify robust clusters without specifying a predetermined number of clusters (Ester et al., 1996). DBSCAN reduced the 41 metabolites included in the final dataset (Fig. S2) into seven high-density clusters (Fig. 4A) with profiles peaking early (coloured blue in Fig. 4B), steadily decreasing or peaking late in diapause (coloured green in Fig. 4B), with the remaining showing more variable patterns of generally increasing concentration (coloured red and yellow in Fig. 4B). Of these, metabolites belonging to the cluster that peaks around 100 days are of greatest interest (dark green profile in Fig. 4A,B), as this is when diapause is terminated (in response to low temperature stimulus) in the studied population (Lehmann et al., 2016). Two-way ANOVA was used to identify nine metabolites for which the profile shapes differed significantly between the treatment groups (Fig. 4A). Of these, aspartate and alanine showed clear peaks, but only alanine differed significantly both in terms of concentration (logFC column in Fig. 4A) as well as profile (profile difference column in Fig. 4A) between the diapause termination and maintenance groups. These concentration differences in alanine (Fig. 4C, left panel) were also significantly different when standardized values were used (Fig. 4C, right panel).

DISCUSSION

The metabolome of *P. napi* is much more dynamic and exhibits a stronger dependence on temperature compared with our earlier analyses of the lipidome (Lehmann et al., 2016, 2017). Because it is known that low temperatures are needed for diapause termination (Posledovich et al., 2015), we contrasted temperature regimes during diapause to identify elements of diapause that might be related to diapause timing processes. The following discussion is partitioned into four parts: cold tolerance, energy metabolism, diapause termination and alanine metabolism.

Cold tolerance

An increase in cold tolerance is generally associated with diapause induction and development (Pullin et al., 1991, 1996; Denlinger and Lee, 2010). We found that supercooling capacity (SCP) and cold shock tolerance in *P. napi* increased more in pupae destined for diapause than in direct developing pupae and, importantly, that pupae moved to cold (diapause terminating) conditions had cold shock tolerance on par with that of pupae kept under warm (diapause maintaining) conditions (Fig. 1A,D). Therefore, developmental path, rather than low temperature acclimation, leads to the initial increase in diapause-associated cold tolerance (Hodkova and Hodek, 2004). After diapause termination, cold shock tolerance decreased during post-diapause development, but only after pupae were taken out of the cold (i.e. after 144 days). This means cold tolerance is maintained during post-termination quiescence but breaks down during ontogenic development after temperatures increases, when the diapause trajectory becomes increasingly similar to the direct development trajectory.

Because the SCP was between −20 and −25°C, and the estimated lower lethal temperature with 50% mortality was between −15 and −20°C in the two diapause measurement points (24 and 114 days) (Fig. 1), *P. napi* represents a freeze-avoiding species that survives by supercooling body fluids during winter (Bale, 1993). Although freeze tolerance was not explicitly tested, all pupae were kept at approximately 2°C for 2 weeks after the SCP measurement, and all of these died during that period, further indicating freeze intolerance. Chronic exposures to mild cold seems to be tolerated as well, as 60% of pupae survive a 30-day exposure to −10°C, indicating that *P. napi* is not chill susceptible (P.L., unpublished

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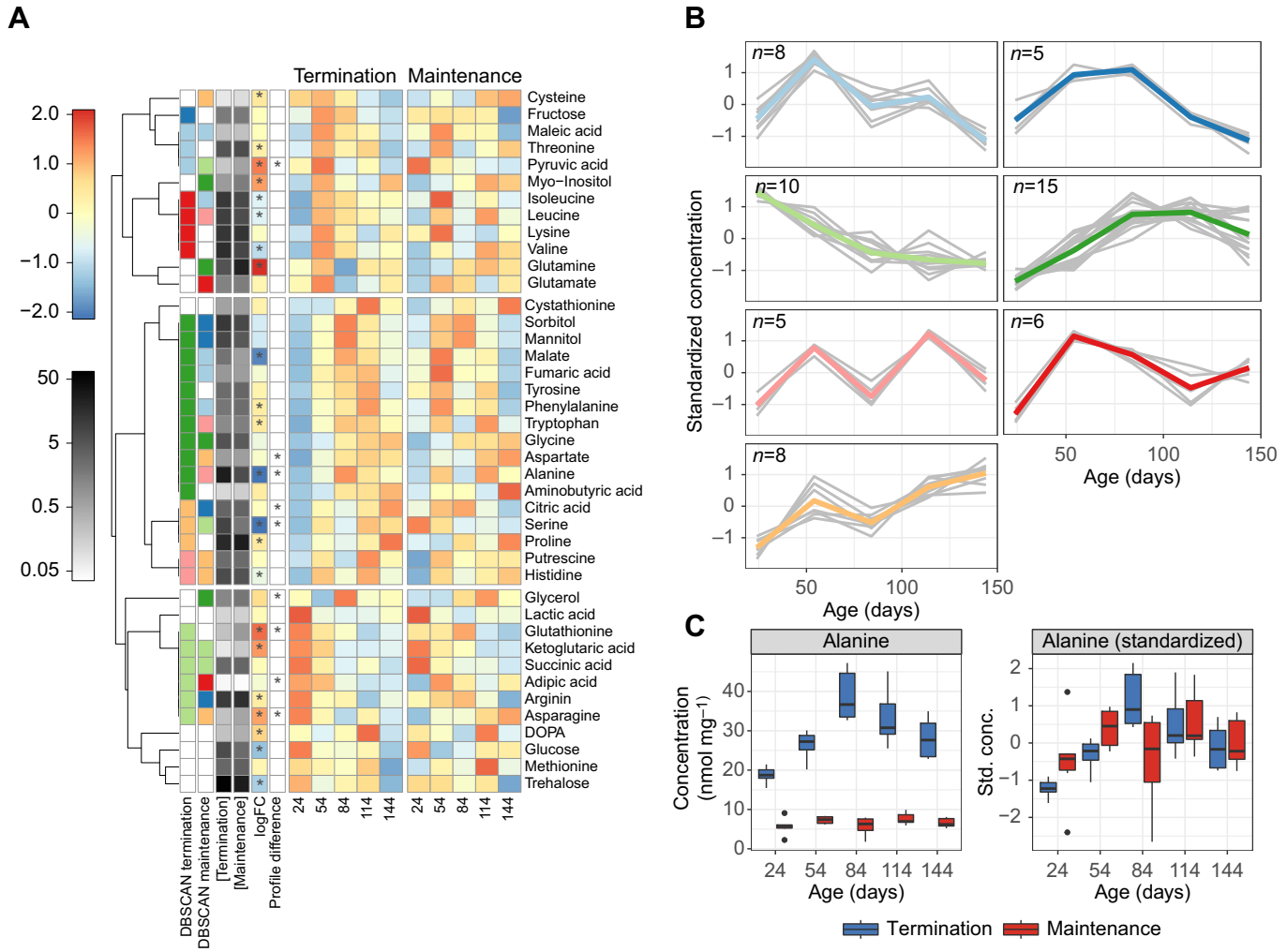

Fig. 4. Metabolomics of diapause termination in *Pieris napi*. (A) Metabolite profiles (mean standardized concentrations in nmol mg^{-1} per time point and treatment group) are shown for each metabolite in the heatmap. Clustering of these profiles was performed using DBSCAN. The resulting seven clusters are shown by colour in the two first annotation columns, for diapause termination and maintenance, respectively (non-clustered metabolites are shown in white). A single metabolite can have different profiles under maintenance versus termination conditions, and the panels in B plot metabolite profiles under both conditions. The third and fourth annotation columns show average (non-standardized) concentrations per metabolite and treatment group, indicating their relative abundance. The fifth annotation column shows $\log_2$ fold-change between treatment groups, and significant differences are indicated with an asterisk (Mann–Whitney *U*-test, adj. $P<0.05$). The sixth column reports two-way ANOVA results comparing profiles between the treatment groups by testing for significant interactions between time, when viewed as a categorical variable, and treatment group ($P<0.05$). (B) Number of individual metabolite profiles (i.e. per metabolite and treatment group) for each of the seven DBSCAN clusters, shown by grey lines. In addition, average cluster profiles are shown as coloured lines to give a general idea of the profile trends per cluster. (C) The boxplots summarize all data points for alanine (raw concentration left, and standardized right) per time point and treatment group. Alanine differed between treatment groups both in raw as well as standardized concentrations, and was the only metabolite that differed in concentration and had a peak-shaped profile. Similar plots for the remaining metabolites are shown in Fig. S2.

observations). The conditions used in this chronic cold tolerance trial, as well as the cold-shock tolerance conditions, are unlikely to reflect the entire variability experienced in the field, so caution must be used for inferences to natural situations (Marshall and Sinclair, 2015). Nevertheless, given that cold tolerance does not show any significant difference between diapause pupae kept at diapause-maintaining and -terminating temperatures, the differences in metabolome between them do not appear related to cold tolerance.

The metabolome generally showed extensive synthesis of metabolites that have been suggested, in mostly correlative experiments, to function as cryoprotectants (Storey and Storey, 1990). These did not include the ubiquitous cryoprotectant glycerol (Pullin and Bale, 1989). Interestingly, sorbitol, which has been shown to be accumulated in a temperature-dependent fashion in previous studies (Storey and Storey, 1983; Michaud and Denlinger, 2007), accumulated to high concentrations in *P. napi*, regardless of temperature (Fig. S2). Pupae accumulated equal total amounts of amino acids in diapause-terminating and -maintaining conditions (Fig. 2B). Sugars, however, were accumulated at much higher concentrations in termination than maintenance pupae (Fig. 2A), even though no clear difference in SCP or cold shock tolerance was observed. Thus, while it is tempting to conclude that free amino acids are the primary drivers of SCP and cold shock tolerance in *P. napi*, and that sugars were accumulated for reasons other than cryoprotection, these conclusions are likely too bold based on the current data. Instead, the accumulation of amino acids suggests that slow breakdown of some dispensable larval protein might be a common feature of pupal diapause metabolism. The pool of free amino acids might later be recruited for rapid synthesis of heat-shock proteins upon sudden, both high and low, thermal shocks.

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Furthermore, free amino acids are likely also part of the slow diapause energy metabolism (more on this below). Sugars are likely synthesized and accumulated primarily at the expense of glycogen (not measured in the present study). In conclusion, because of the relatively low concentrations of total accumulated metabolites in the diapause termination group, their colligative effects on melting point and supercooling capacity are likely limited (Zachariassen, 1985). However, it is still likely that they have other cold-tolerance-promoting effects, such as stabilization of protein and membrane structures (Storey and Storey, 1990).

Energy metabolism

Another potential explanation for the differences in the metabolome between the diapause maintenance and termination pupae could relate to energy metabolism. A previous study found that while the respiratory quotient (RQ) clearly suggests lipid metabolism during winter, lipid stores decreased minimally during the 5-month study period (Lehmann et al., 2016). Therefore, other metabolic processes were likely confounding the results. One explanation might be extensive energy production through anaerobic metabolism, observed in several diapausing insects, such as *Sarcophaga crassipalpis* (Michaud and Denlinger, 2007) and *Wyeomyia smithii* (Emerson et al., 2010), or as a response to thermal stress, such as in *Dinocras cephalotes* (Verberk et al., 2013). While many intermediates of the citric acid cycle (e.g. fumarate and citrate) increased throughout diapause, there were also some signs of active glycolysis, such as increased levels of pyruvic acid throughout winter. Therefore, there is no strong evidence for stalling of the tricarboxylic acid (TCA) cycle during diapause. Because no clear conclusion on energy production can be drawn, it is important to note that because diapause is a strongly hypometabolic state in *P. napi* (Lehmann et al., 2016), the small energy demands might be masked by other ongoing processes as long as energy production is sufficient. Thus, the low ATP demand during diapause seems to be sufficiently supplied from a mix of resources where lipids dominate (Lehmann et al., 2016). Proteins and glycogen seem to primarily serve in building the cryoprotectant profile, discussed above. The accumulated amino acids and sugars are finally converted to energy upon resumption of development in spring (Fig. 2), and as such they are not lost but represent an alternative form of substrate storage during winter (Storey and Storey, 1990).

As the diapause maintenance pupae were kept at 20°C for prolonged periods, high membrane fluidity (Hazel, 1995) could result in the leakage of metabolites into the interstitial fluid and a pathological state. Such a scenario could explain why so many of the metabolites that showed clear dynamics in the diapause termination pupae did not show any pattern (or high variability) in the diapause maintenance pupae (Fig. S2). However, because diapause often is induced early in summer in *P. napi* from the current population (Lehmann et al., 2016), warm temperatures, especially early in diapause, do not represent an unnatural environmental setting.

Diapause termination

Because the end of diapause is relatively well characterized in the studied population (Posledovich et al., 2015; Lehmann et al., 2016, 2017), we specifically sampled pupae flanking the time when the termination process is complete and diapause (sometimes referred to as endogenous diapause) transitions into post-diapause quiescence. Generally, three scenarios on cold-exposure-mediated diapause termination can be envisioned. During cold periods, (1) certain metabolite(s) build up and diapause is terminated when they reach above a critical level; (2) metabolites are depleted, and diapause is terminated when they fall to sufficiently low levels; or (3) a combination of the above happens: some need to accumulate, while others need to deplete. In our data, metabolites belonging to the dark blue, dark green and yellow cluster (Fig. 4B) could be associated with the first scenario. Metabolites belonging to the light blue, light green or dark red cluster (Fig. 4B) could be associated with the second scenario.

One interesting candidate was sorbitol, which in *B. mori* (Chino, 1958) acts according to the second scenario described above, i.e. maintaining diapause. Also, in the current experiment, sorbitol (together with mannitol) exhibited a conspicuous pattern (Fig. 4, Fig. S2), with a clear peak around the time of diapause termination. However, because the analyses could not firmly determine whether this pattern differs from those in diapause maintenance pupae, the results indicate that sorbitol dynamics (as well as other shared metabolites, Fig. 4B) are not linked to diapause termination in *P. napi*. Instead, metabolites that differed between the diapause maintenance and termination groups are of interest (Fig. 4A). Although nine metabolites differed between the diapause maintenance and termination pupae, we argue that the most relevant metabolite is alanine, because it shows a clear peak in termination pupae, but none whatsoever in maintenance pupae. Like with sorbitol and mannitol, this peak coincided with the time at which diapause is terminated in the studied population (Lehmann et al., 2017a). Alanine is accumulated during diapause in many insects, and has been suggested to work as a cryoprotectant (Goto et al., 2001; Michaud and Denlinger, 2007). Here we suggest that alanine might have other functions in diapause than promoting cold tolerance in *P. napi*, as SCP and cold shock tolerance did not differ between the groups, even though alanine concentration differed dramatically. Because alanine showed a peak and pyruvate showed the opposite pattern, the data suggest direct synthesis of alanine from pyruvate in the termination pupae. Whether alanine (1) is causally maintaining endogenous diapause until termination, (2) is a developmental block but downstream of diapause terminating factors, (3) reflects a metabolic switch or process linked to low temperature that is not visible in warm diapause pupae or (4) is a metabolic component of post-termination physiology (that is, completely unrelated to diapause termination itself) remains unclear.

Alanine metabolism

What is clear is that at some point during diapause termination in *P. napi*, rates of alanine catabolism overtake rates of alanine anabolism. There are two main biochemical scenarios that could explain this pattern. The most plausible (1) is partial anaerobiosis, where the rate of glycolysis is slightly faster than the rate of the TCA cycle due to low ATP turnover, which likely is linked to ‘double’ metabolic suppression, by both diapause and low temperatures. In this situation, glycolysis still supplies the building blocks for production of putative cryoprotectants (e.g. mannitol, sorbitol and other sugars). Excess pyruvate may then be diverted from the TCA cycle to alanine formation through a transamination reaction which, at the same time, converts glutamate to ketoglutarate (Nation, 2015). Ketoglutarate then enters the TCA cycle, produces one GTP directly, and its carbon skeleton may end up partially in aspartate and later asparagine and thus partially may be used to slowly rotate the TCA cycle. As stated above, energy metabolism, even though heavily suppressed, remains aerobic during diapause in *P. napi* (Lehmann et al., 2016). Another scenario (2) is when proline acts as energy substrate and is converted to ketoglutarate via glutamate,

again involving the transamination of pyruvate to alanine (Nation, 2015). This is known to occur mainly in active insects and processes of high energy demand, such as insect flight (Beenakkers et al., 1984; Scaraffia and Wells, 2003). Again, a source of pyruvate is needed, which could be supplied from glycolysis, but also from the digestion of other amino acids. The original source of proline in that case is (in addition to stored proline) extracellular protein (e.g. extracellular matrix, such as collagen), which is digested by metalloproteinases (i.e. collagenases). Because we can see a gradual increase of proline and glycine (the most abundant amino acids of collagen) and some other amino acids, this could indicate that the extracellular matrix is gradually degraded during diapause in *P. napi*. Although both of these scenarios could explain the peak of alanine (as well as some other of the observed patterns), a firm determination of their relative contributions, and their links to diapause termination at low temperature, needs further study.

In *B. mori*, a cold-inducible gene called *Samui* is implicated as the link between low temperatures and sorbitol dehydrogenase expression that ultimately reduces sorbitol concentration and leads to diapause termination (Moribe et al., 2001). Further studies in *P. napi* should screen for cold-induced expression of metabolic genes involving alanine metabolism. A different mechanism could relate to expression of termination-related genes through alanine-sensitive promoter activation. In *Arabidopsis* plants, a mechanism involving the proline-sensitive sequence ACTCAT in promoters has been shown to regulate expression of osmoprotection-related genes (Satoh et al., 2002; Oono et al., 2003). This is an important potential mechanism to study further in animals. Finally, there are also links between metabolome dynamics and epigenetic modifications, which have been implicated in diapause, for example, in the wasp *Nasonia vitripennis* (Pegoraro et al., 2016). For instance, ketoglutarate is an important co-factor of dioxygenase enzymes (Wu et al., 2016), which in turn are involved, among other things, in epigenetic modifications (Breiling and Lyko, 2015). Thus, without a sufficient ketoglutarate concentration, there could be restrictions in the extent of possible epigenetic modifications. Because ketoglutarate is involved in both the biochemical scenarios outlined in the previous paragraph, similar substrate limitations that regulate epigenetic control mechanisms in *N. vitripennis* might involve alanine in *P. napi*.

Conclusions

The present study investigates metabolome dynamics during diapause in *P. napi*. We find that cold tolerance develops, at least partially, without cold-priming, and that, contrary to the notion of a metabolically inactive state, diapause is characterized by massive temperature-dependent and -independent changes in the profiles of metabolites across several metabolic pathways. Metabolites grouped into distinct temporal profiles: some steadily increased throughout diapause, other decreased throughout diapause and some showed a peak that coincided with diapause termination. Because pupae were reared under conditions either promoting or inhibiting diapause termination, we were able to target nine profiles that differed depending on termination status. Of these, alanine was particularly interesting as a diapause-termination-associated candidate because it accumulated to high concentrations in the termination group, degraded in a temperature-independent fashion mid-winter, but did not accumulate at all in the maintenance group. Further experiments, including manipulations or population comparisons, are needed to further investigate the roles of these metabolites. If not mechanistically related to diapause termination (Xu et al., 2012), they can still be very useful as biochemical markers of this important developmental transition. In general, we suggest that more studies focusing on potential regulatory roles of metabolites are needed, based on the data presented here. Finally, this study highlights the importance of moving from binary design (i.e. diapause versus direct) to one incorporating time-series sampling when studying dynamic metabolic processes such as diapause. Without the time-series design across different treatments, we could not have identified termination-specific patterns in the metabolome, highlighting the advance of our design compared with previous large-scale analytic approaches, such as RNA-seq, overcoming previously acknowledged design limitations (Ragland and Keep, 2017).

Competing interests

The authors declare no competing or financial interests.

Author contributions

Conceptualization: P.L., V.K., S.N., C.W., C.W.W., K.G.; Methodology: P.L., P.P., V.K., M.M., P.S., S.N., R.A., L.V., C.W., C.W.W., K.G.; Software: R.A., L.V.; Formal analysis: P.L., P.S., R.A., L.V., K.G.; Investigation: P.L., P.P., V.K., M.M., R.A., L.V., C.W., K.G.; Resources: P.L., P.P., M.M., P.S., S.N., K.G.; Data curation: P.L., V.K., M.M., P.S., R.A., L.V.; Writing - original draft: P.L., P.P., V.K., S.N., R.A., L.V., C.W., C.W.W., K.G.; Writing - review & editing: P.L., P.P., V.K., R.A., L.V., C.W., C.W.W., K.G.; Visualization: P.L., P.P., R.A., K.G.; Supervision: V.K.; Project administration: P.L., S.N., C.W.W., K.G.; Funding acquisition: V.K., P.S., S.N., C.W.W., K.G.

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Data availability

Full data are available from the Dryad Digital Repository (Lehmann et al., 2017b): https://doi.org/10.5061/dryad.f0dg5

Supplementary information

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References

Bale, J. S. (1993). Classes of insect cold hardiness. *Funct. Ecol.* **7**, 751-753.
Beenakkers, A. M. T., Van der Horst, D. J. and Marrewijk, W. J. A. (1984). Insect flight muscle metabolism. *Insect Biochem.* **14**, 243-260.
Breiling, A. and Lyko, F. (2015). Epigenetic regulatory functions of DNA modifications: 5-methylcytosine and beyond. *Epigenetics Chromatin* **8**, 24.
Chino, H. (1958). Carbohydrate metabolism in diapause eggs of the silkworm, Bombyx mori. II. Conversion of glycogen into sorbitol and glycerol during diapause. *J. Insect Physiol.* **2**, 1-12.
Danks, H. V. (2007). The elements of seasonal adaptations in insects. *Can. J. Entomol.* **139**, 1-44.
Denlinger, D. L. and Lee, R. E. J. (2010). *Low Temperature Biology of Insects*. Cambridge: Cambridge University Press .
Denlinger, D. L., Yocum, G. D. and Rinehart, J. P. (2012). Hormonal control of diapause. In *Insect Endocrinology* (ed. L. I. Gilbert), pp. 430-464. London: Academic Press.
Emerson, K. J., Bradshaw, W. E. and Holzapfel, C. M. (2010). Microarrays reveal early transcriptional events during the termination of larval diapause in natural populations of the mosquito (*Wyoeomyia smithii*). *PLoS ONE* **5**, e9574.
Ester, M., Kriegel, H., Sander, J. and Xu, X. (1996). A density-based algorithm for discovering clusters in large spatial databases with noise. *Proceedings of the Second International Conference on Knowledge Discovery and Data Mining*, pp. 226-231.
Friberg, M., Aalberg Haugen, I. M., Dahlerus, J., Gotthard, K. and Wiklund, C. (2011). Asymmetric life-history decision-making in butterfly larvae. *Oecologia* **165**, 301-310.
Goto, M., Li, Y.-P., Kayaba, S. & Outani,S. and Suzuki,K. (2001). Cold hardiness in summer and winter diapause and post-diapause pupae of the cabbage armyworm, *Mamestra brassicae* L. under temperature acclimation. *J. Insect Physiol.* **47**, 709-714.

Gotthard, K. and Nylin, S. (1995). Adaptive plasticity and plasticity as an adaptation: a selective review of plasticity in animal morphology and life history. *Oikos* **74**, 3-17.
Hahn, D. A. and Denlinger, D. L. (2007). Meeting the energetic demands of insect diapause: Nutrient storage and utilization. *J. Insect Physiol.* **53**, 760-773.
Hazel, J. R. (1995). Thermal adaptation in biological membranes: Is homeoviscous adaptation the explanation? *Annu. Rev. Physiol.* **57**, 19-42.
Hodek, I. (1996). Diapause development, diapause termination and the end of diapause. *Eur. J. Entomol.* **93**, 475-487.
Hodek, I. (2002). Controversial aspects of diapause development. *Eur. J. Entomol.* **99**, 163-173.
Hodkova, M. and Hodek, I. (2004). Photoperiod, diapause and cold-hardiness. *Eur. J. Entomol.* **101**, 445-458.
Horie, Y., Kanda, T. and Mochida, Y. (2000). Sorbitol as an arrester of embryonic development in diapausing eggs of the silkworm, *Bombyx mori*. *J. Insect Physiol.* **46**, 1009-1016.
Hušek, P. and Šimek, P. (2001). Advances in amino acid analysis. *LC-GC North America* **19**, 986-999.
Jiang, H., Wei, Z., Nachman, R. J. and Park, Y. (2014). Molecular cloning and functional characterization of the diapause hormone receptor in the corn earworm *Helicoverpa zea*. *Peptides* **53**, 243-249.
Kiguchi, K., Kitazawa, T. and Ohtsuki, Y. (1990). In vitro culture of the dechorionated eggs in the silkworm, *Bombyx mori* Linne′: development of the embryos recovered from the culture media. *Proc. Arthropoda Embryological Soc. Japan* **25**, 15-17.
Koštál, V., Korbelová, J., Rozsypal, J., Zahradníčková, H., Cimlová, J., Tomčala, A. and Šimek, P. (2011). Long-term cold acclimation extends survival time at 0°C and modifies the metabolomic profiles of the larvae of the fruit fly *Drosophila melanogaster*. *PLoS ONE* **6**, e25025.
Koštál, V., Korbelová, J., Štětina, T., Poupardin, R., Colinet, H., Zahradníčková, H., Opekarová, I., Moos, M. and Šimek, P. (2016). Physiological basis for low-temperature survival and storage of quiescent larvae of the fruit fly *Drosophila melanogaster*. *Sci. Rep.* **6**, 32345.
Lehmann, P., Lyytinen, A., Piiroinen, S. and Lindström, L. (2015). Latitudinal differences in diapause related photoperiodic responses of European Colorado potato beetles (*Leptinotarsa decemlineata*). *Evol. Ecol.* **29**, 269-282.
Lehmann, P., Pruisscher, P., Posledovich, D., Carlsson, M., Käkelä, R., Tang, P., Nylin, S., Wheat, C. W., Wiklund, C. and Gotthard, K. (2016). Energy and lipid metabolism during direct and diapause development in a pierid butterfly. *J. Exp. Biol.* **219**, 3049-3060.
Lehmann, P., Van der Bijl, W., Nylin, S., Wheat, C. W. and Gotthard, K. (2017a). Timing of diapause termination in relation to variation in winter climate. *Physiol. Entomol.* **42**, 232-238.
Lehmann, P., Pruisscher, P., Kostal, V., Moos, M., Simek, P., Nylin, S., Agren, R., Varemo, L., Wiklund, C., Wheat, C. W. and Gotthard, K. (2017b). Data from: Metabolome dynamics of diapause in the butterfly Pieris napi: distinguishing maintenance, termination and post-diapause phases. Dryad Digital Repository. doi:10.5061/dryad.f0dg5
Marshall, K. E. and Sinclair, B. J. (2015). The relative importance of number, duration and intensity of cold stress events in determining survival and energetics of an overwintering insect. *Funct. Ecol.* **29**, 357-366.
Michaud, M. R. and Denlinger, D. L. (2007). Shifts in the carbohydrate, polyol, and amino acid pools during rapid cold-hardening and diapause-associated cold-hardening in flesh flies (*Sarcophaga crassipalpis*): a metabolomic comparison. *J. Comp. Physiol B* **177**, 753-763.
Moribe, Y., Niimi, T., Yamashita, O. and Yaginuma, T. (2001). Samui, a novel cold-inducible gene, encoding a protein with a BAG domain similar to silencer of death domains (SODD/BAG-4), isolated from *Bombyx* diapause eggs. *Eur. J. Biochem.* **268**, 3432-3442.
Nation, J. L. (2015). *Insect Physiology and Biochemistry*. Boca Raton: CRC Press.
Nelson, R. J., Denlinger, D. L. and Somers, D. E. (2010). *Photoperiodism, the Biological Calendar*. New York: Oxford University Press.
Oono, Y., Seki, M., Nanjo, T., Narusaka, M., Fujita, M., Satoh, R., Satou, M., Sakurai, T., Ishida, J., Akiyama, K. et al. (2003). Monitoring expression profiles of Arabidopsis gene expression during rehydration process after dehydration using ca.7000 full-length cDNA microarray. *Plant J.* **34**, 868-887.
Pegoraro, M., Bafna, A., Davies, N. J., Shuker, D. M. Tauber, E. (2016). DNA methylation changes induced by long and short photoperiods in *Nasonia*. *Genome Res.* **26**, 203-210.
Posledovich, D., Toftegaard, T., Wiklund, W., Ehrlén, J. and Gotthard, K. (2015). Latitudinal variation in diapause duration and post-winter development in two pierid butterflies in relation to phenological specialization. *Oecologia* **177**, 181-190.
Pruisscher, P., Larsdotter-Mellström, H., Stefanescu, C., Nylin, S., Wheat, C. W. and Gotthard, K. (2017). Sex-linked inheritance of diapause induction in the butterfly *Pieris napi*. *Physiol. Entomol.* **42**, 257-265.
Pullin, A. S. (1996). Physiological relationships between insect diapause and cold tolerance: coevolution or coincidence? *Eur. J. Entomol.*, **16**, 121-129.
Pullin, A. S. and Bale, J. S. (1989). Influence of diapause and temperature on cryoprotectant synthesis and cold hardiness in pupae of *Pieris brassicae*. *Comp. Biochem. Physiol. A* **94**, 499-503.
Pullin, A. S., Bale, J. S. and Fontaine, X. L. R. (1991). Physiological aspects of diapause and cold tolerance during overwintering in *Pieris brassica*. *Physiol. Entomol.* **16**, 447-456.
Ragland, G. J. and Keep, E. (2017). Comparative transcriptomics support evolutionary convergence of diapause responses across Insecta. *Physiol. Entomol.* **42**, 246-256.
Satoh, R., Nakashima, K., Seki, M. and Shinzoaki, K. (2002). ACTCAT, a novel cis-acting element for proline- and hypoosmolarity-responsive expression of the *ProDH* gene encoding proline dehydrogenase in *Arabidopsis*. *Plant Physioloy.* **130**, 709-719.
Scaraffia, P. Y. and Wells, M. A. (2003). Proline can be utilized as an energy substrate during flight of *Aedes aegypti* females. *J. Insect Physiol.* **49**, 591-601.
Sinclair, B. J., Coello Alvarado, L. E. and Ferguson, L. V. (2015). An invitation to measure insect cold tolerance: methods, approaches, and workflow. *J. Thermal Biol.* **53**, 180-197.
Sokal, R. R. and Rohlf, F. J. (2003). *Biometry, the Principles and Practice of Statistics in Biological Research*. New York: W.H. Freeman and Company.
Stålhandske, S., Lehmann, P., Pruisscher, P. and Leimar, O. (2015). Effect of winter cold duration on spring phenology of the orange tip butterfly, *Anthocharis cardamines*. *Ecology Evolution* **5**, 5509-5520.
Storey, J. M. and Storey, K. B. (1983). Regulation of cryoprotectant metabolism in the overwintering gall larva *Eurosta solidaginis*: temperature control of glycerol and sorbitol levels. *J. Comp. Physiol. B* **149**, 495-502.
Storey, K. B. and Storey, J. M. (1990). Biochemistry of cryoprotectants. In *Insects at Low Temperature* (ed. R. E. J. Lee and D. L. Denlinger), pp. 64-94. New York: Chapman and Hall
Tauber, M. J., Tauber, C. A. and Masaki, S. (1986). *Seasonal Adaptations of Insects*. New York: Oxford University Press.
Verberk, W. C. E. P., Sommer, U., Davidson, R. L. and Viant, M. R. (2013). Anaerobic metabolism at thermal extremes: a metabolomic test of the oxygen limitation hypothesis in an aquatic insect. *Integr. Comp. Biol.* **53**, 609-619.
Williams, C. M., McCue, M. D., Sunny, N. E., Szejner-Sigal, A., Morgan, T. J., Allison, D. B. and Hahn, D. A. (2016). Cold adaptation increases rates of nutrient flow and metabolic plasticity during cold exposure in *Drosophila melanogaster*. *Proc. R. Soc. B* **283**, 20161317.
Wu, L.-F., Meng, S. and Tang, G. (2016). Ferrous iron and α-ketoglutarate-dependent dioxygenases in the biosynthesis of microbial natural products. *Biochimica et Biophysica Acta* **1864**, 453-470.
Xu, W.-H., Lu, Y.-X. and Denlinger, D. L. (2012). Cross-talk between the fat body and brain regulates insect developmental arrest. *Proc. Natl. Acad. Sci. USA* **109**, 14687-14692.
Yocum, G. D., Rinehart, J. P. and Larson, M. L. (2011). Monitoring diapause development in the Colorado potato beetle, *Leptinotarsa decemlineata*, under field conditions using molecular biomarkers. *J. Insect Physiol.* **57**, 645-652.
Zachariassen, K. E. (1985). Physiology of cold tolerance in insects. *Physiol. Rev.* **64**, 799-832.
Zhang, T. Y., Sun, J.-S., Zhang, L. B., Shen, J.-L. and Xu, W.-H. (2004). Cloning and expression of the cDNA encoding the FXPRL family of peptides and a functional analysis of their effect on breaking pupal diapause in *Helicoverpa armigera*. *J. Insect Physiol.* **50**, 25-33.