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RESEARCH ARTICLE

Energy and lipid metabolism during direct and diapause development in a pierid butterfly

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ABSTRACT

Diapause is a fundamental component of the life cycle in the majority of insects living in environments characterized by strong seasonality. The present study addresses poorly understood associations and trade-offs between endogenous diapause duration, thermal sensitivity of development, energetic cost of development and cold tolerance. Diapause intensity, metabolic rate trajectories and lipid profiles of directly developing and diapausing animals were studied using pupae and adults of *Pieris napi* butterflies from a population in which endogenous diapause has been well studied. Endogenous diapause was terminated after 3 months and termination required chilling. Metabolic and post-diapause development rates increased with diapause duration, while the metabolic cost of post-diapause development decreased, indicating that once diapause is terminated, development proceeds at a low rate even at low temperature. Diapausing pupae had larger lipid stores than the directly developing pupae, and lipids constituted the primary energy source during diapause. However, during diapause, lipid stores did not decrease. Thus, despite lipid catabolism meeting the low energy costs of the diapausing pupae, primary lipid store utilization did not occur until the onset of growth and metamorphosis in spring. In line with this finding, diapausing pupae contained low amounts of mitochondria-derived cardiolipins, which suggests a low capacity for fatty acid β-oxidation. While ontogenic development had a large effect on lipid and fatty acid profiles, only small changes in these were seen during diapause. The data therefore indicate that the diapause lipidomic phenotype is developed early, when pupae are still at high temperature, and retained until post-diapause development.

KEY WORDS: Fatty acids, Lipid stores, *Pieris napi*, Pupa, Respirometry, Stress

INTRODUCTION

Synchronization of the life cycle to seasonal variation is a central aspect of the life history strategy of most organisms. In strongly seasonal environments this has led to the evolution of adaptations that allow both survival in harsh conditions and an efficient use of periods when growth and reproduction are possible. Diapause is a 'pre-programmed arrested state of development' common in organisms that cannot remain active or undertake long-range migration during periods of seasonal stress (Tauber et al., 1986) and consists of several phases that can be grouped into three stages (Koštál, 2006): induction, maintenance and termination. In many insects, the induction of diapause is facultative, allowing them to produce one or several directly developing generations followed by diapause in the last generation of the season (Tauber et al., 1986). After induction, most of diapause is spent in diapause maintenance, a phase subdivided into endogenous diapause and post-diapause quiescence (or exogenous diapause). During endogenous diapause, insects are non-responsive to external signals, and only after diapause is terminated, often during mid-winter, and insects shift to post-diapause quiescence (Hodek, 1996, 2002) do they become sensitive to external signals (e.g. temperature, photoperiod or humidity), which can mediate post-diapause development (Koštál, 2006). While it is well established that the correct timing of diapause termination is critical for winter survival and post-winter fitness (Tauber and Tauber, 1976; Danks, 1987), the ecological and physiological mechanisms of diapause termination are unclear (Hodek, 1996, 2002; Koštál, 2006), especially compared with diapause induction (Danilevskii, 1965; Bradshaw and Holzapfel, 2007; Nelson et al., 2010).

Energy must be stored and used sparingly not only to maintain essential body functions during diapause but also for post-diapause demands (Hahn and Denlinger, 2007). While important, associations and trade-offs between diapause termination and energetics remain relatively poorly understood (Hahn and Denlinger, 2011). For instance, is the primary role of stored energy to sustain animals during endogenous diapause or post-diapause quiescence, or alternatively to fuel post-diapause development or even post-diapause processes (e.g. adult fecundity)? Do energy sources change as diapause progresses? If energy is used to maintain animals in diapause, then what are the energetic costs of prolonged periods of post-diapause quiescence and thermal conditions during endogenous diapause (Williams et al., 2012)?

In an attempt to provide insights into the aforementioned aspects of diapause, the present study investigated the association between endogenous diapause duration, thermal sensitivity of endogenous and post-diapause development and lipid dynamics during diapause. Ambient temperature plays an important role in all of these stages, but in different ways. During early diapause, before temperatures decrease in autumn, insensitivity to temperatures promoting post-diapause development is important to avoid premature development (Tauber and Tauber, 1976). During diapause, chilling may be needed for endogenous diapause to progress to termination and post-diapause quiescence (Tauber et al., 1986; Hodek and Hodková, 1988), in a fashion similar to the much better understood vernalization process in plants (Brunner et al., 2014). In ectotherms, low temperatures are associated with low metabolic rates (MRs), which are beneficial for the energetics of overwintering (Irwin and Lee, 2003). However, low temperatures lead to rigidity of cell membranes, and to uphold homeostasis,

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List of symbols and abbreviations

BMC	Bonferroni multiple comparison
CL	cardiolipin
CLI	chain length index
DAG	diacylglycerol
DB	daily energy budget
FA	fatty acid
GLM	generalized linear models
MR	metabolic rate
PC	phosphatidylcholine
PCA	principal component analysis
PE	phosphatidylethanolamine
PI	phosphatidylinositol
PS	phosphatidylserine
Q_{10}	temperature coefficient
RH	relative humidity
RQ	respiratory quotient
SM	sphingomyelin
TAG	triacylglycerol
UI	unsaturation index
U:S	unsaturation ratio

membrane lipids are often restructured as part of diapause development in order to maintain the proper physiological semifluid state of the membrane (Koštál, 2010). Finally, post-diapause development is temperature dependent, and temperature variation is important for synchronizing adult emergence (Stålhandske et al., 2015). Here, the diapause biology of *Pieris napi* (Linnaeus) (Lepidoptera: Pieridae), a temperate butterfly in which pupal diapause is facultatively induced, was studied in comparison with direct (non-diapause) development.

First, we investigated the length of endogenous diapause, maximal diapause duration and post-diapause development of *P. napi* pupae. We hypothesized that (1) chilling is needed to break diapause and that (2) increasing time spent at low temperature accelerates post-diapause development. Next, we studied the MR of pupae undergoing either direct or diapause development. We hypothesized that (3) MR is heavily suppressed during diapause and that (4) temperature sensitivity of MR is low during endogenous diapause. In addition, we determined the chemical form, size and depletion patterns of energy stores in pupae undergoing direct and diapause development. We hypothesized that (5) lipids constitute the main energy store and that (6) they are depleted in a linear fashion during diapause. Finally we characterized the whole-body lipidome of pupae and adult *P. napi* as they progress through major developmental transitions. Our general hypotheses were that diapause leads to (7) membrane lipid remodeling, that (8) ontogenic and diapause development have a major impact on the lipidome and that (9) choice of developmental pathway leaves a physiological mark on adults emerging from either pathway.

MATERIALS AND METHODS

Animals and general rearing protocol

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. Butterflies of both sexes (P1) were kept in cages (0.8×0.8×0.5 m) and provided with a host plant, *Armoracia rusticana* Gaertner, Meyer & Scherbius (Brassicales: Brassicaceae) for oviposition at 25°C and 60% relative humidity (RH) under long-day conditions (next to large windows and under 400 W metal halide lamps) and fed sugar water. Their offspring (F1) were mass-reared to adulthood on *A. rusticana* on a long-day photoperiod (22 h light:2 h dark, 20°C, 80% RH) and re-mated under similar conditions to their parents. For the experiments, the F2 generation was reared to pupation in groups of five on *A. rusticana* leaves under conditions inducing either diapause (10 h light:14 h dark, 20°C, 80% RH) or direct development (22 h light:2 h dark, 20°C, 80% RH) in climate cabinets (KB8400L, Termaks, Bergen, Norway). Diapausing pupae were then divided randomly into four experimental groups: diapause intensity, direct development, diapause and warm diapause (Fig. 1; Table S1).

Diapause intensity

To estimate the length of endogenous diapause, assess maximal diapause duration and investigate post-diapause development in the sampled population, diapausing pupae were sexed and divided into four diapause duration treatments (Fig. 1). Pupae were kept for 2, 4, 6 or 12 months at 2°C before being moved to 20°C. According to previous research (Posledovich et al., 2015), the 2 month point should precede diapause termination, while the 4 month and later time points should represent post-termination where development has probably already resumed. Pupae were weighed to the nearest 0.1 mg (Sauter RE1614) and placed individually into 20 ml syringes that acted as respirometry chambers throughout the experiment. For the first day of respirometry measurement, pupae were kept at 2°C. Then pupae were moved to 20°C and re-measured every 24 h, until the day they eclosed. If no development was seen after 14 days at 20°C, the pupae were considered to be still in endogenous diapause and were only measured after 30 and 45 days to confirm that no development had occurred.

Dynamic injection respirometry

CO_2 production and O_2 consumption of individual pupae of both sexes were measured throughout direct development and diapause at 2 and 20°C (Fig. 1) to assess MR. Pupae were weighed to the nearest 0.1 mg (Sauter RE1614) and placed in 20 ml syringes (i.e. respirometry chambers). The syringes were filled with air that was first scrubbed of CO_2 with ascarite (Thomas Scientific, Swedesboro, NJ, USA) and then passed through filtered acidified water (pH<4.5, checked weekly), closed with three-way luer valves, and kept for roughly 24 h at the appropriate temperature (20, 10 or 2°C). An empty syringe served as a control. CO_2 production was measured using a Sable Systems (Las Vegas, NV, USA) differential respirometry setup. Two independent lines of outdoor air scrubbed of H_2O and CO_2, using Drierite (W. A. Hammond, Xenia, OH, USA) and ascarite, respectively, were pushed at a steady rate of 150 ml min^{-1} using an SS-4 pump (Sable Systems) and two separate mass flow controllers (840 Series; Sierra Instruments Inc., Monterey, CA, USA). Syringes containing pupae were placed after the mass valve controllers in the first line (sample) and 17 ml was pushed into the airflow. The push rate was recorded through a second flowmeter downstream of the syringe and approximated a flow rate of 162 ml min^{-1} downstream of the syringe. The line was then scrubbed of H_2O with Drierite and entered the sample line of a Li-7000 CO_2 analyzer (LiCor, Lincoln, NB, USA). The second line (reference) proceeded the same way, mimicking the exact length of the sample line (including an empty measurement chamber), entering the reference line of the CO_2 analyzer. The lines then proceeded through a second set of ascarite CO_2 scrubbers and entered an Oxzilla FC-2 O_2 analyzer (Sable Systems). Preliminary

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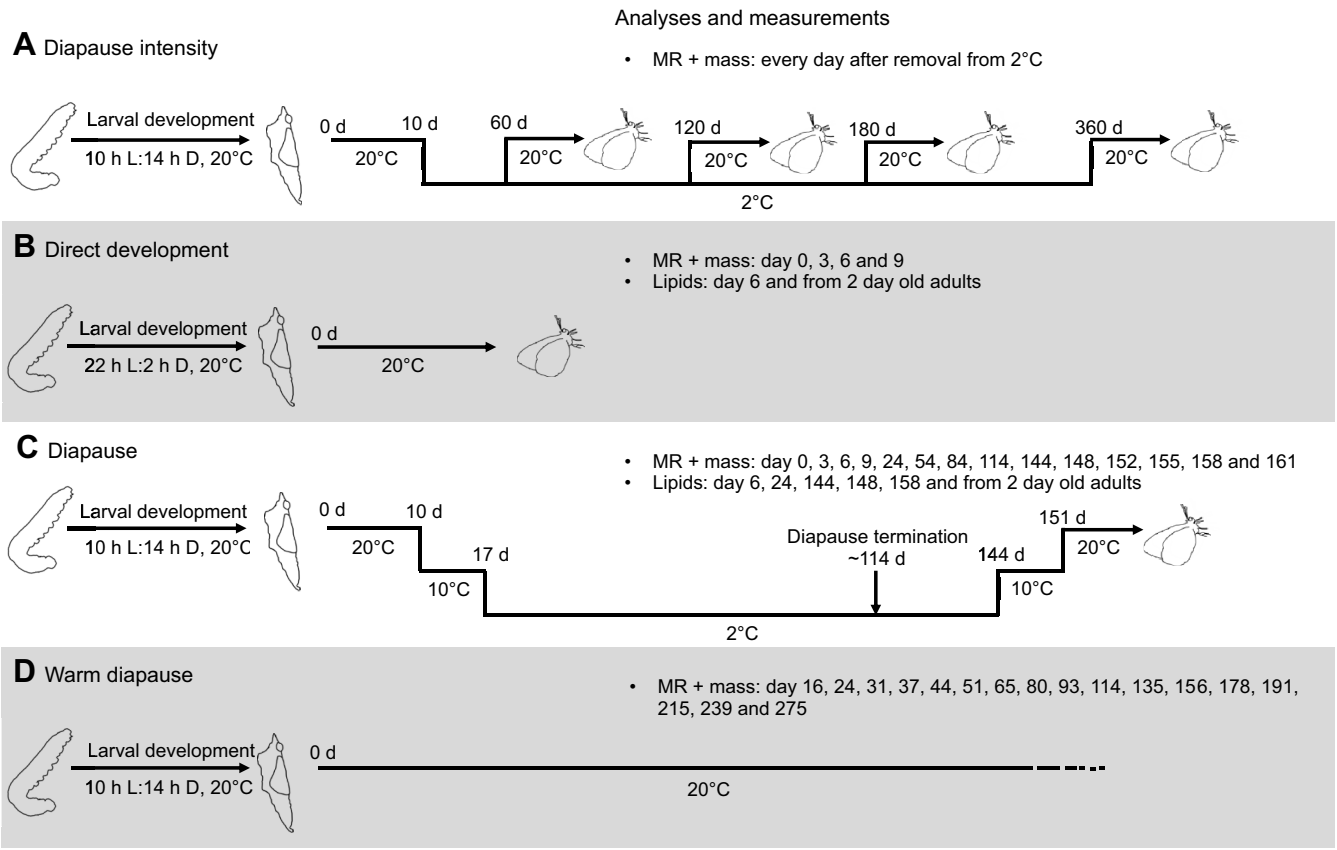

Fig. 1. Overview of treatments and analyses in the current study on lipid metabolism and energetics of direct development and diapause in *Pieris napi*. Each panel reflects one treatment and starts with larval rearing conditions (L, light; D, dark). After pupation [0 days (d)], pupae were maintained under four different conditions: (A) diapause intensity, (B) direct development of pupae, (C) pupae undergoing cold diapause and (D) pupae kept at a warm temperature during diapause. Transitions are written above the lines and reflect the number of days after pupation at which a change in holding temperature occurred, while the corresponding temperature is shown below the lines. Sampling points for the analyses are given ('Analyses and measurements'). MR, metabolic rate. For exact sample sizes, see Table S1.

measurements were performed to ensure stability of flow rate through either channel by measuring the flow rate of air ejected from the O_2 analyzer. Differential CO_2 and O_2 were calculated by subtracting the output of the reference line from the output of the sample line. For all measurements, sampling rate was 1 Hz. In the program Expedata (version 1.7.30), the raw output was baseline corrected against the reference line value, fractioned and multiplied by flow rate to yield CO_2 and O_2 in ml min^{-1} (Lighton, 2008). Finally, the values were corrected by subtracting the readings from the empty control syringe from the sample values.

MR was calculated by first integrating CO_2 and O_2 against time to yield, in ml, CO_2 produced and O_2 consumed while pupae were in the syringes. Then, $\dot{V}_{CO_2}$ and $\dot{V}_{O_2}$ were corrected by accounting for the fraction of air that was still left in the syringe and the time spent in the syringe using the formula (for $\dot{V}_{CO_2}$): $\dot{V}_{CO_2}$=[CO_2×(20/17)]/hours in syringe (Lighton, 2008). Then, the respiratory quotient (RQ) was calculated as: RQ=$\dot{V}_{CO_2}/\dot{V}_{O_2}$ (Schmidt-Nielsen, 1991). Finally, MR (in W=J s^{-1}) was converted from $\dot{V}_{O_2}$ using the formula: MR={$\dot{V}_{O_2}$×[16+(5.164×RQ)]}/(60×60) (Lighton, 2008). The energy budget of development after post-diapause pupae were moved to 20°C was then calculated. First MR (in W) was converted to daily energy budget (in J): DB=MR×86,400 (s day^{-1}). Then, the DB values until hatching were summed to produce total energy (in J), interpreted as a measure of developmental budget after being moved to 20°C. Finally, the temperature coefficient (Q_{10}) for MR was calculated as: $Q_{10}=(R_2/R_1)^{10/(T_2-T_1)}$ where R reflects MR at temperature T. In the present study, T_2 was 20°C and T_1 was 2°C, and the sampling point was at 80 days into diapause.

Whole-body lipidomics

Sampling points for the lipidomic analysis were chosen based on the respirometry data and the diapause intensity experiment to reflect major developmental transitions (Fig. 1). At each point, three males and three females were sampled. Adults were kept singly after hatching, 22 h light:2 h dark, 20°C, 80% RH, in climate cabinets (KB8400L, Termaks, Bergen, Norway) and fed only water. Sampling day 24 reflects early endogenous diapause, day 144 reflects post-diapause quiescence, day 148 reflects early post-diapause development after 3 days at 10°C, and day 158 reflects later post-diapause development after 7 days at 10 and 7 days at 20°C (see Table S1). The status of pupae as developing or not was verified through respirometry as described above (data not shown).

Total lipids were extracted according to Folch et al. (1957). For mass spectrometry (MS), extract aliquots were dissolved in chloroform/methanol 1:2 (by volume) and spiked with a cocktail of 14 internal standards. Just prior to MS analysis, 1% NH_4OH was added and the sample solutions were infused to the electrospray source of a triple quadrupole mass spectrometer (Agilent 6490 Triple Quad LC/MS with iFunnel technology; Agilent Technologies, Inc., Santa Clara, CA, USA) at a flow rate of 8 µl min^{-1}. Nitrogen was used as the nebulizing (20 psi), drying (11 µl min^{-1} at 250°C) and collision gas. Positive and negative ion

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modes were used, and phospholipid species were selectively detected using head-group-specific MS/MS scanning modes (Brügger et al., 1997) with 25–65 eV collision energy (optimal settings depend on the lipid class). Phosphatidylcholine (PC), lysoPC and sphingomyelin (SM) species were detected as precursors of *m/z* 184 (P184). Phosphatidylethanolamine (PE) and lysoPE species were detected by using their neutral loss of 141 (NL141), phosphatidylserine (PS) species as NL87 and phosphatidylinositol (PI) species as P241. Triacylglycerol (TAG) and diacylglycerol species (DAG) were detected in the positive ion mode as $(M^+NH_4)^+$ ions (Duffin et al., 1991) and cardiolipin (CL) in the negative ion mode as doubly charged ions. Mass spectra were processed by MassHunter software (Agilent Technologies, Inc.) and individual lipid species were quantified by using internal standards and LIMSA software (Haimi et al., 2006) and expressed in mole percentage (mol %). Lipid species were abbreviated as: [chain total carbon number]:[chain total number of double bonds]. Relative concentrations of lipid classes were obtained by summing up the concentrations of the individual lipid species in a class.

The fatty acids (FAs) in the total lipids were determined as methyl ester derivatives by gas chromatography employing standard protocols (e.g. Käkelä et al., 2005). The quantitative composition was analyzed by a Shimadzu GC-2010 Plus machine with flame-ionization detector, and identification of FA structures was performed by Shimadzu GCMS-QP2010 Ultra with a mass-selective detector. Zebron ZB-wax capillary columns (30 m, 0.25 mm i.d. and film thickness 0.25 µm; Phenomenex, Torrence, CA, USA) and helium as carrier gas were used. The FA composition was calculated as mol %, and FAs were marked by using the abbreviation: [carbon number]:[number of double bonds]n-[position of the first double bond calculated from the methyl end] (e.g. 22:6n-3).

Several indexes related to lipid viscosity were calculated, such as ratio of PE to PC (PE/PC), ratio of unsaturated to saturated FAs (unsaturation ratio, U:S) and average number of double bonds per FA (unsaturation index, UI, or double bond index). The latter is defined as: UI=Σ(% monounsaturated FAs)+(2×% diunsaturated FAs) (etc.)/100 (Kates, 1986), and the UI was also applied to specific phospholipid classes. In addition, the ratio of FAs with 16 carbons to FAs with 18 carbons (C16/C18) and chain length index (CLI) were calculated. The latter is defined as CLI=Σ(12×% C12 FAs)+(13×% C13 FAs) (etc.)/100. CLI was especially well suited to demonstrating a change in species composition of SM, containing few unsaturated molecules.

Statistical analyses

Because some traits did not meet the assumptions of normality of error distribution and homogeneity of variance and were uncorrectable through transformations, generalized linear models (GLM) were used throughout for statistical analyses. In the text below, mass is fresh mass in grams, age is days since pupation, and pathway is direct or diapause development, unless stated otherwise. In all tests, a fully factorial design was used. Non-significant interactions and main effects were removed from final models (Sokal and Rohlf, 2003) and model improvement was tracked through the Akaike information criterion. In the case of significant effects, *post hoc* comparisons of main level effects were performed with Bonferroni multiple-group corrections. All statistical tests were performed with the IBM SPSS statistics 23.0 (IBM SPSS Inc., Chicago, IL, USA) software package.

The effect of time in diapause on hatching age after removal from the cold, MR at 2°C, the slope of the MR trajectory during development (see Table S2 for curve-fitting metrics) and energy expenditure during development were in all cases analyzed with starting body mass, months in diapause and sex as explanatory variables. The effect of pathway on MR during days 0–10 was analyzed with mass, sex, age and pathway as explanatory variables. A similar model was used to test the difference in MR of 80 day old

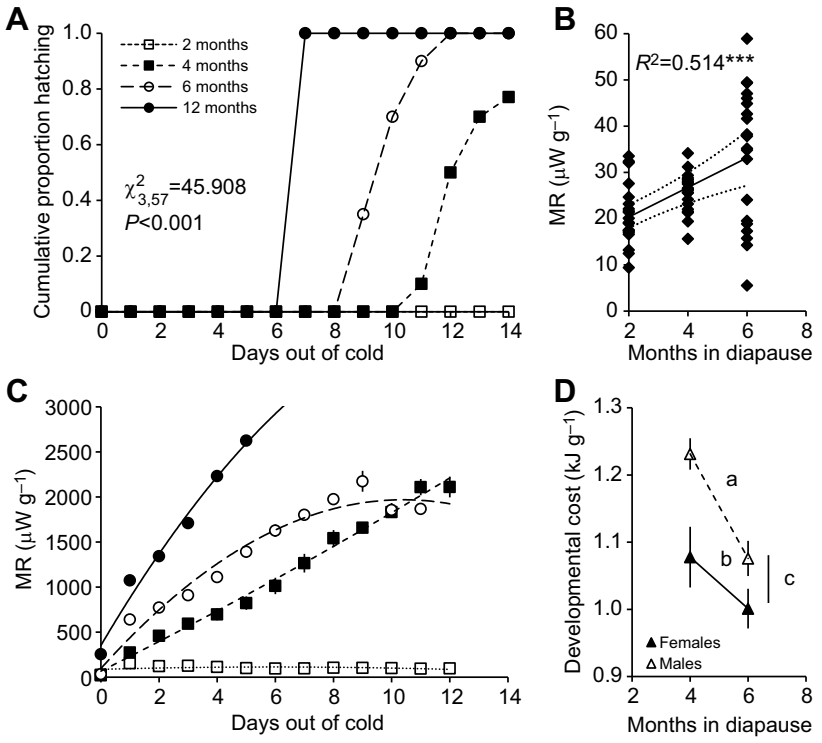

Fig. 2. Diapause intensity and post-diapause development in *P. napi*. (A) The cumulative proportion of hatched pupae after different periods of time spent in diapause as a function of the number of days after removal from the cold. The results of a chi-square test are shown, where the number of months in diapause was arrayed against the number of pupae remaining in diapause or developing. (B) Diapause MR. ***P<0.001. (C) MR (means±s.e.) as a function of the number of days after removal from the cold. The overall effect of the number of months in diapause on the slope of the MR trajectory was highly significant (P<0.001, Table 1C). (D) An estimate of the cost of development (means±s.e.) of pupae after 4 and 6 months in diapause. Letters a and b indicate significant (P<0.05) differences between months within sex while c indicates an overall difference between the sexes. In A–C, N=20 per month (only a single pupa survived till hatching in the 12 month group; in the other month groups, mortality was very low); in D, N=10 per point.

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Table 1. Diapause intensity

Effect	Wald χ^2	d.f.	Significance
A: Hatching age			
Intercept	165.434	1	<0.001
Mass	2.170	1	0.141
Month	61.576	1	<0.001
B: Diapause MR			
Intercept	1.628	1	0.202
Mass	8.660	1	0.003
Month	20.889	1	<0.001
C: Slope of MR trajectory			
Intercept	24.346	1	<0.001
Month	223.171	1	<0.001
D: Energy budget of development			
Intercept	4.457	1	<0.001
Mass	6.090	1	<0.001
Sex	10.002	1	0.002
Month	88.048	1	0.014

Results of generalized linear models (GLM) investigating the effects of time in diapause on post-diapause development and physiology in *Pieris napi*. For the slope of the metabolic rate (MR) trajectory, the slope of a quadratic function explaining the effect of the number of days out of the cold on MR was first calculated for each individual, and then used as a dependent variable. A–D correspond to panels A–D in Fig. 2

Table 2. Diapause energetics

Effect	Wald χ^2	d.f.	Significance
MR – direct versus diapause during first 10 days			
Intercept	2.143	1	0.143
Mass	9.228	1	0.002
Age	126.612	3	<0.001
Pathway	232.347	1	<0.001
Age×pathway	193.976	3	<0.001
MR – warm versus cold diapause at 80 days			
Intercept	0.019	1	0.889
Mass	3.860	1	0.049
Sex	4.059	1	0.044
Pathway	124.773	1	<0.001
Sex×pathway	7.727	1	0.005
TAG content – direct versus diapause			
Intercept	6750.850	1	<0.001
Group	1087.982	7	<0.001
Sex	14.116	1	<0.001
Group×sex	40.152	7	<0.001
RQ – direct versus diapause			
Intercept	6473.233	1	<0.001
Age	1.705	1	0.192
Pathway	3.713	1	0.054
RQ – comparison between major transitions			
Intercept	7480.833	1	<0.001
Group	195.578	2	<0.001

Results of GLM investigating differences in MR between *P. napi* pupae undergoing direct versus diapause development, and diapause at 2 versus 20°C at 80 days. Triacylglycerol (TAG) content was compared between male and female pupae undergoing direct or diapause development. Differences in respiratory quotient (RQ) between pupae undergoing direct development and diapause development are shown, as well as between different phases of development or diapause (direct 0–10 days, diapause 0–10 days, diapause 24–144 days and post-diapause development 148–161 days).

pupae between cold and warm diapause treatments. The effect of time in warm diapause on mass and MR was analyzed with the logarithm of mass and MR as dependent variables, while the logarithm of age, the squared logarithm of age and sex were used as explanatory variables. The quadratic term was added as curve fitting suggested that a quadratic function best explained the patterns. The effect of pathway and age on RQ, TAG content and functional lipid traits was analyzed with age and pathway as explanatory variables. A second test of differences in RQ between three age groups: days 0–10 (pathways pooled), 24–144 and 148–161, was also performed with RQ as the dependent variable and age group as the factorial explanatory variable. Principal component analyses (PCA) on mole percentages of lipid classes, lipid species and fatty acids were performed to visually present age- and pathway-dependent changes. Loaded compounds can be seen in Tables S3 and S4. The major principal component extracted through PCA was then added as a dependent variable in a GLM, with age and pathway as explanatory variables.

RESULTS

Diapause intensity

There was a strong effect of time at 2°C on overall hatching propensity (Fig. 2A). After 2 months (60 days), no hatching occurred when pupae were moved to 20°C, indicating this time point still represents endogenous diapause. After 4 months (120 days), 77% hatched, and after 6 months (180 days), all pupae hatched, suggesting that diapause is terminated between 2 and 4 months at 2°C in this population. After 12 months (360 days), mortality was very high (96%), but the single pupa that was still alive hatched. In the other cohorts, mortality was very low (<6%). These data suggest that the maximal diapause duration was shorter than or around 12 months. The time required to hatch after removal from the cold decreased with time spent at 2°C (Table 1A, Fig. 2A), indicating that development had already started at some time point during winter at 2°C. Although no clear morphological signs of development (e.g. wing formation) were observed in the 2, 4 and 6 month pupae when taking them out of the 2°C environment, we found that MR measured at 2°C on day 0 (i.e. before pupae were moved to warmer conditions) increased with time spent at 2°C (Table 1B, Fig. 2B). This indicates a cumulative effect of time in winter on metabolic or developmental processes. After moving pupae to the higher temperature (20°C), metabolic trajectories increased in a non-linear fashion with age, with a slope that increased with time spent at 2°C (Table 1C, Fig. 2C). The DB of pupae after they were moved to 20°C decreased with time spent at 2°C and this cost was higher in males than in females (Table 1D, Fig. 2D).

MR trajectories of pupae undergoing direct or diapause development

There was a strong overall effect of developmental pathway on MR (Table 2). In both pathways, MR decreased between day 0 and day 3; however, after this time point the pathways diverged (Table 2, Fig. 3A). In pupae undergoing direct development, MR increased exponentially until adult eclosion at around 10 days. In contrast, in pupae undergoing diapause development, MR decreased. After 24 days (10 days at 2°C), MR was suppressed at a level retained throughout the overwintering period (Fig. 3B). Also, in warm diapause pupae, MR was suppressed after day 10 and remained at a low level throughout diapause (Fig. 3B). However, as these pupae were kept at a higher temperature than those undergoing diapause at 2°C, their MR was significantly higher (Fig. 3C, Table 2). At 144 days (approximately 4.5 months), pupae were moved to higher temperatures and MR showed an exponential increase, as also observed in the diapause intensity experiment (Fig. 2C). In the warm diapause pupae, no exponential increase in MR was observed at any point (Fig. 3B), indicating that chilling is needed for endogenous diapause to proceed to diapause termination and subsequent exogenous diapause. Nevertheless, MR also increased

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Table 3. Warm diapause MR and mass loss

Effect	Coefficient	s.e.	Wald χ^2	d.f.	Significance
MR					
Intercept	2.865	0.424	45.653	1	<0.001
Sex	–0.132	0.027	24.900	1	<0.001
Age	–2.158	0.467	21.370	1	<0.001
Age squared	0.637	0.125	25.853	1	<0.001
Mass					
Intercept	0.057	0.037	2.434	1	0.119
Sex	–0.007	0.002	8.085	1	0.004
Age	0.120	0.041	8.852	1	0.003
Age squared	–0.038	0.011	12.284	1	<0.001

Coefficients of GLM investigating MR trajectory mass loss in *P. napi* pupae subjected to warm diapause. Age refers to the base 10 logarithm of days since pupation. Quadratic functions best explained the relationship between response variables and age.

with time in warm diapause pupae (Table 3). Furthermore, both the increase in MR and the decrease in mass followed non-linear patterns (Table 3, Fig. 3D,E). The point at which the curve changed shape probably reflects a point when endogenous diapause cannot be maintained any longer at high temperature. Interestingly, these pupae still did not hatch after this point.

Size of lipid reserves and respiratory quotient

Diapausing pupae had a high TAG content, around 70% of total lipids (Fig. 4A). Male pupae had lower TAG levels than female pupae and the levels varied between the developmental pathways and with age (Table 2). Comparison of 6 day old pupae showed that diapausing pupae had a significantly higher TAG content than those undergoing direct development (roughly 70% versus 50%; Fig. 4A). Interestingly, no significant decrease in TAG content was seen during diapause. Even after 5 months at 2°C, the TAG content was as high as in 6 day old diapausing pupae (Fig. 4A). After moving the pupae to 20°C, however, a dramatic decrease in TAG content could be seen accompanying development, and indeed 158 day post-diapause pupae, after spending 7 days at 10°C and 7 days at 20°C, showed the same TAG content as 6 day directly developing pupae. Overall TAG content was lowest in adults, and at this stage there was no difference between butterflies that developed with or without diapause.

In parallel with TAG content, the RQ of pupae was estimated. No difference between the pathways in RQ was seen during the first 10 days (Table 2). RQ averaged around 0.75 during this period. After 10 days, RQ decreased to around 0.55 and remained low throughout diapause (Fig. 4B), until pupae were moved to 20°C to increase post-diapause development rate (day 144). Thereafter, RQ rapidly increased to levels similar to those of the early diapause period at 20°C (Table 2). Average RQ during diapause was significantly lower in the cold diapause than in the warm diapause group (0.519±0.026 and 0.718±0.012, respectively, means±s.e.; univariate GLM: Wald χ^2=63.487, d.f.=1, P<0.001).

Lipidome dynamics

Alterations of the whole lipidome related to diapause or direct development of the butterflies were first analyzed by PCA, which revealed three major patterns detected at the level of lipid class, lipid species and FA composition (Table 4). First, major shifts in the profiles were associated with ontogenic developmental stages, which

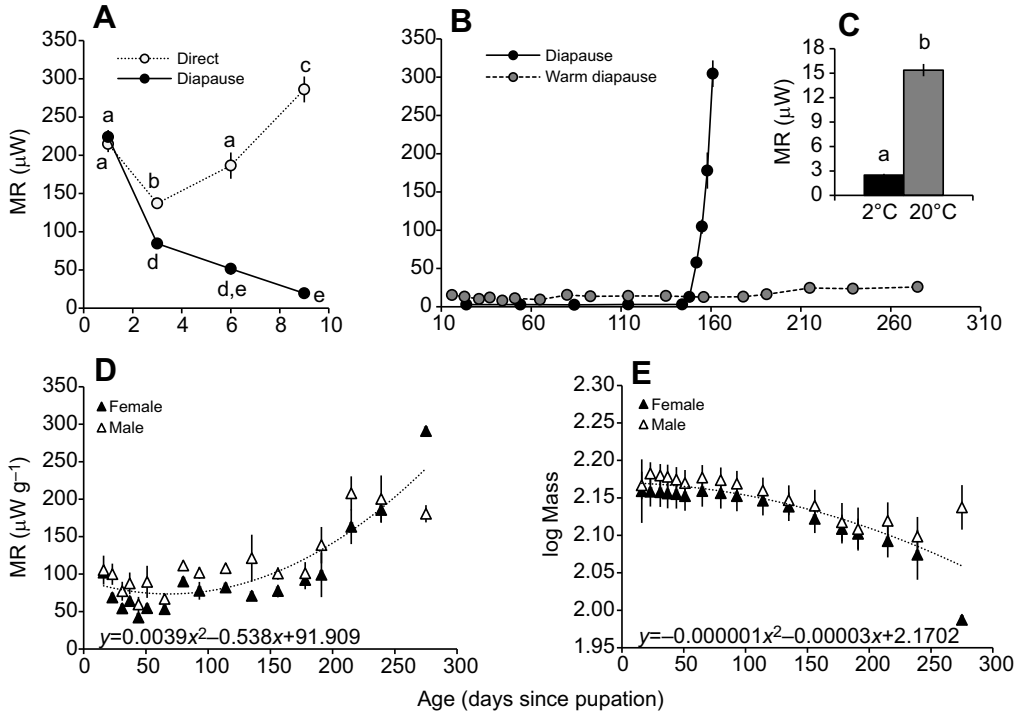

Fig. 3. MR and mass loss in *P. napi* pupae undergoing direct or diapause development at high or low temperature. (A) MR (means±s.e.) during the first 10 days for pupae undergoing direct and diapause development. Different letters denote significant Bonferroni-corrected between-group comparisons (P<0.05). (B) MR (means±s.e.) during diapause in pupae kept at 2 or 20°C. At 144 days, pupae from the diapause group were moved to 20°C for diapause termination. (C) MR (means±s.e.) of pupae in diapause at 2 or 20°C at 80 days. Different letters denote significant Bonferroni-corrected between-group comparisons (P<0.05). (D) MR (means±s.e.) and (E) mass (mg; means±s.e.) of pupae in warm diapause. In both D and E, quadratic functions (dotted line of combined data) best explained the data. Even though mass-specific data are plotted in D, the analysis was performed with mass as covariate. For direct and diapause pupae, N=20–25 per point; N=12 pupae were repeatedly measured in the warm diapause group (Table S1D).

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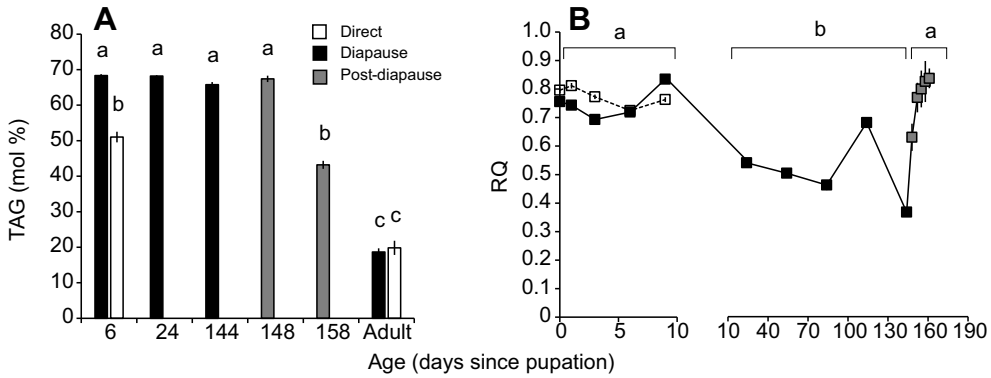

Fig. 4. Lipid storage and utilization in *P. napi* undergoing direct or diapause development. (A) Differences in lipid store (triacylglycerol, TAG) size and utilization (means±s.e.) between pupae undergoing direct and diapause development. (B) Differences in respiratory quotient (RQ; means±s.e.) between the groups. Different letters denote significant Bonferroni-corrected between-group comparison differences (P<0.05). In B, between-group comparisons were performed on pooled data from the diapausing 0–10, 24–144 and 148–161 day old individuals. Pathways did not differ in RQ during days 0–10 (see Table 2). In A, N=6 per column; in B, N=20–25 per point (Table S1B,C).

constituted the major explanatory axis (PCA1) in all PCA analyses. This axis explained 61% of variation in lipid class composition, 39% in individual lipid species composition and 51% in FA composition (Fig. 5). Second, no major shifts in these profiles were seen throughout diapause and post-diapause development at low temperature (from day 6 to 148), indicating that diapause did not induce any large-scale changes in lipid metabolism, either those related to energy production or changes indicating membrane remodeling. Third, no significant differences were observed in lipid class profiles between samples representing the respective developmental stages in directly developing and diapausing pupae (6 day direct versus 158 day post-diapause, and adults; Table 4). In lipid species and FA profiles, some minor differences between the samples were found (Fig. S1, S2; Tables S3, S4).

Lipid class profile

Lipid class profiles largely differed according to developmental stage, but showed less variation at the same time point when comparing the two pathways (Fig. 5A; Fig. S1A). In diapausing pupae, the major lipid class was TAG, which made up about 70% of total lipid content, followed by the main phospholipids PE and PC (10% and 8%, respectively). DAG and SM constituted about 5%, while PI and PS made up about 2–3% of total lipids in all stages. In adults, TAG content was about 20% of total lipids, significantly lower than in pupae, while PE, PC, lysoPE and CL TAG content (30%, 20%, 10% and 10%, respectively) was significantly higher than that of the pupae. The developing stages, 6 day old direct pupae and 158 day old post-diapause developing pupae, showed lipid class profiles that were intermediate to the adult and 2–4 month old diapause pupae profiles.

Lipid species profile

For lipid species profiles, major transitions also entailed developmental shifts from diapausing pupae to adults (Table 4, Fig. 5B; Fig. S2). Adult lipid species profiles were characterized by elevated levels of highly unsaturated lipid species, especially

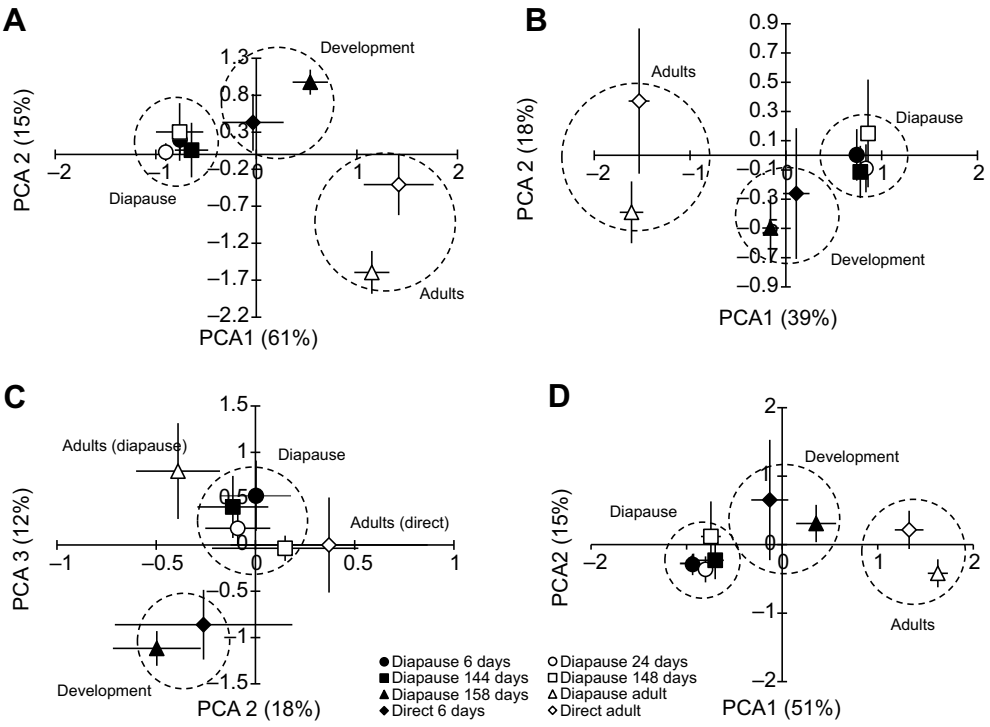

Fig. 5. Principal component analyses representing overall changes in whole-body lipid profiles in *P. napi* undergoing direct or diapause development. (A) Lipid class, (B,C) lipid species (1 and 2, respectively) and (D) fatty acid (FA) composition between individuals undergoing direct or diapause development. Age is given as the number of days since pupation. In A, B and D, PCA1 is plotted against PCA2; in C, PCA2 is plotted against PCA3 (means±s.e.). Dashed circles have been drawn arbitrarily around groups that represent the three major developmental states used in the comparisons (diapausing pupae, developing pupae and adults). The lipids and FAs loaded into the analyses are given in Tables S3 and S4. See Results and Table 4 for statistical significance. N=6 per point except for direct development at 6 days, where N=5.

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Table 4. Diapause lipidomics

Effect	Wald χ^2	d.f.	Significance
Lipid class – PCA1 (61%)			
Intercept	0.033	1	0.856
Age	118.651	5	<0.001
Pathway	6.272	1	0.012
Lipid species – PCA1 (39%)			
Intercept	0.293	1	0.588
Age	1057.728		<0.001
Pathway	14.158	1	<0.001
Age×pathway	23.119	1	<0.001
Fatty acids – PCA1 (60%)			
Intercept	0.124	1	0.725
Age	398.851	5	<0.001
Pathway	4.365	1	0.037
Age×pathway	20.835	1	<0.001
PC/PE			
Intercept	4103.686	1	<0.001
Group	44.407	7	<0.001
U:S			
Intercept	9128.136	1	<0.001
Group	78.894	7	<0.001
UI			
Intercept	38,029.257	1	<0.001
Group	18.828	7	0.009
UI of PC			
Intercept	70,696.863	1	<0.001
Group	634.816	7	<0.001
UI of PE			
Intercept	38,029.257	1	<0.001
Group	18.828	7	<0.001
C16/C18			
Intercept	2779.100	1	<0.001
Group	120.273	7	<0.001
CLI of SM			
Intercept	487,924.838	1	<0.001
Group	734.858	7	<0.001

Results of GLM investigating differences in lipid class, lipid species and fatty acid profiles as well as traits related to membrane function between *P. napi* pupae undergoing direct and diapause development. The major explanatory axis (PCA1) of a principal component analysis on all compounds was used as the dependent variable for lipid class, lipid species and fatty acid profiles. Between-group comparisons of PC/PE, U:S, UI, UI of PC, UI of PE, C16/C18 and CLI of SM are shown in Fig. 6.

PC, phosphatidylcholine; PE, phosphatidylethanolamine; U:S, unsaturation ratio; UI, unsaturation index; C16/C18, ratio of 16 carbon fatty acids to 18 carbon fatty acids; CLI, chain length index; SM, sphingomyelin.

TAG54:7 (isobaric species having 18:1n-9/18:3n-3/18:3n-3 as the main individual molecular species), PE36:6 (18:3n-3/18:3n-3 as the only acyl pair) and PC36:6 (18:3n-3/18:3n-3) (Fig. S2A–C). Among SM species, mostly composed of saturated species, adults had longer acyl chains than the pupae (Fig. S2D). The 6 day direct developing pupae differed significantly from all points [Bonferroni multiple comparison (BMC): $P<0.001$] except the 158 day post-diapause developing pupae (BMC: $P=0.530$), further emphasizing that they represent the same developmental stage (Fig. S2). Diapausing 6 day old pupae did not differ from the 24 or 144 day old diapausing or the 148 day old post-diapausing pupae but were significantly different from all other stages of development (BMC: $P<0.001$). Finally, while slightly separated, lipid species profiles of adults did not differ significantly from each other, regardless of developmental pathway, but differed significantly from all other stages (BMC: $P<0.001$). Unlike lipid class and fatty acid analyses, with two important PCAs explaining the sample composition, the PCA analysis of the lipid species data suggested three axes significantly contributed to lipid species variation. The third axis separated adult and diapausing pupae from developing pupae (Fig. 5C).

FA profiles

Changes found in the FA profiles confirmed the pattern found for lipid class and species profiles (Table 4). The 6 day direct developing pupae differed significantly from all developmental stages, except the 158 day post-diapause developing pupae (BMC: $P<0.001$). Diapausing 6 day old pupae did not differ from the 24 or 144 day old diapausing pupae or the 148 day old post-diapausing pupae but were significantly different from all other time points (BMC: $P<0.001$). Finally, adults did not differ from each other, regardless of developmental pathway, but differed significantly from all other points (BMC: $P<0.001$). The most abundant individual FA was 18:3n-3 (Fig. S1B). Compared with pupae, adults had higher levels of 18:0 and less 16:0. Adults contained very small amounts of 16:3n-3, which was the third main polyunsaturated FA in pupae.

Functional trait analyses

The functional lipid traits calculated from the lipid class and FA data showed similar patterns to the PCA analyses. Generally, very small changes occurred throughout diapause (Table 4) and only adults differed from the other groups. In PE/PC ratio, a significant but transient increase was seen during diapause (Fig. 6A), while an overall decrease in C16/C18 ratio occurred with development (Fig. 6E). Directly developed adults had a significantly lower U:S of total FAs than adults that hatched after diapause (Fig. 6B); otherwise, no difference was seen between adults. When the degree of unsaturation or average chain length was studied in specific membrane phospholipid classes, very pronounced differences were found. At the end of diapause, the UI of PE and PC increased and in adults this index, representing average FA double bond content in these phospholipids, peaked (Fig. 6D). In addition, SM acyl chains of adults got longer at the end of diapause and CLI reached maximum length in adults (Fig. 6F).

DISCUSSION

Diapause intensity

In the present study on *P. napi* from southern Sweden, endogenous diapause was terminated and shifted to post-diapause quiescence (exogenous diapause; Koštál, 2006), after 2–4 months had elapsed at 2°C. The results also suggest that the maximal diapause duration is likely to be under 12 months in the field and are in line with previous data on the same population (Posledovich et al., 2015). Endogenous diapause is important as it decreases the risk of early emergence as a result of heat spells during autumn and early winter months (Danks, 2007). Pupae kept at high temperature also showed a suppression of MR, indicating that chilling is not needed to express the diapause phenotype in this population. However, as none of the warm diapause pupae hatched during the 300 day observation period, they probably remained in endogenous diapause throughout. This strongly suggests that chilling is needed for endogenous diapause to progress to post-diapause quiescence in *P. napi* (Posledovich et al., 2015).

There was a non-linear relationship between mass, MR and time in warm diapause, showing that pupae have a relatively high ability to retain their mass and have a suppressed MR during the first months of endogenous diapause even at high temperature. Because diapause is typically induced well in advance of deteriorating environmental conditions (Nelson et al., 2010), the first months of diapause can be spent at relatively high temperature. As high

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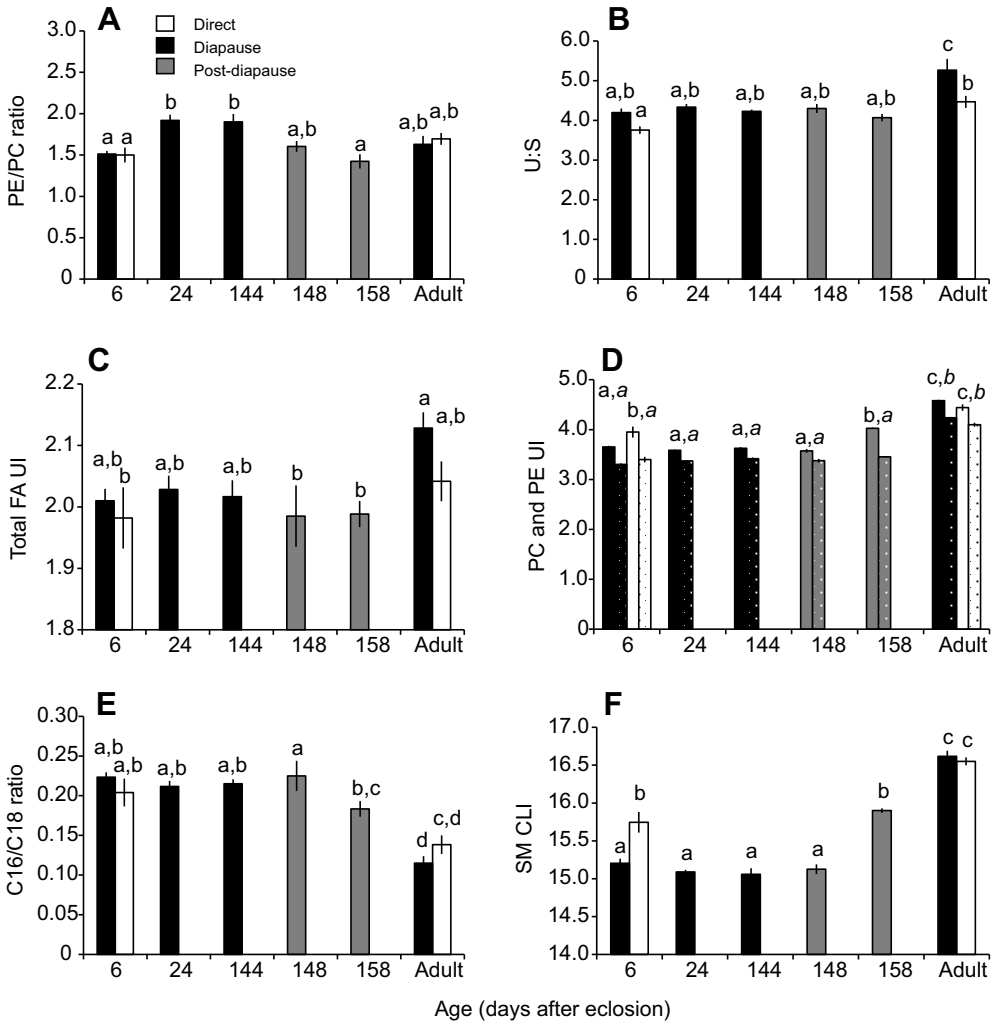

Fig. 6. Overall changes in lipid traits of *P. napi* undergoing direct or diapause development.
(A) Phosphatidylethanolamine/phosphatidylcholine (PE/PC) ratio. (B) Unsaturation ratio (U:S) in total FAs. (C) Unsaturation index (UI) of total FAs. (D) Unsaturation index of PE and PC acyl chains. (E) Ratio of FAs with 16 carbons to FAs with 18 carbons. (F) Chain length index (CLI) of sphingomyelin (SM) acyl chains. Means±s.e. Different letters denote significant Bonferroni-corrected between-group comparisons (P<0.05). Open columns represent individuals undergoing direct development and filled columns represent diapause development. In D, columns depicting PE results are dotted and between-group comparisons are marked with italic letters. N=6 per column, except for direct development at 6 days, where N=5.

temperatures during diapause generally are associated with relatively high energy consumption and poorer post-winter fecundity (Irwin and Lee, 2003; Hahn and Denlinger, 2011), temperature-unrelated suppression of MR during endogenous diapause could be interpreted as an adaptation to save energy during the early part of diapause, before temperatures decrease. Nevertheless, MR is strongly temperature dependent and in the current study Q_{10} calculated during endogenous diapause was 2.65, suggesting that pupae at 20°C have roughly 6 times higher MR than when kept at 2°C. Thus, diapause induction early in summer is probably related to relatively high energy consumption in *P. napi*, and indeed, major energy loss during diapause generally occurs early in diapause (Ushatinskaya, 1956; Williams et al., 2012).

MR

During diapause at 2°C, MR slowly increased, suggesting a cumulative effect of time on some metabolic processes during winter (Hodek and Hodková, 1988). Also, the developmental budget estimated from MR, which is a very rough estimate of post-diapause developmental cost, decreased with time spent in diapause, as did the time required to eclose after removal from winter conditions. Together, the data indicate that post-diapause ontogenic development is initiated even at 2°C. This could be due to a physiological inability to fully suppress development, but it is also possible that this reflects an ecological fail-safe mechanism that optimizes post-winter development time (e.g. Wiklund and Solbreck, 1982). An increase in development rate with time spent in diapause has also been suggested in other butterflies (Wiklund and Solbreck, 1982; Gray, 2009; Xiao et al., 2013; Stålhandske et al., 2015). Also, the rising MR slope reflects an accelerated post-diapause developmental rate with time spent at 2°C. The MR trajectories during post-diapause development after pupae were moved to 20°C were very similar to the MR trajectory of directly developing pupae (Fig. 3A,B). This indicates that diapause reflects a temporal removal from the direct development trajectory, as also seen in, for instance, *Rhagoletis pomonella* (Ragland et al., 2009, 2011).

Directly developing pupae and diapausing pupae showed similarly decreasing MR trajectories after the first days of pupation, which probably reflects apoptotic breakdown of larval tissues (Schmolz and Lamprecht, 2000). After 3 days, the directly developing pupae showed a shift in MR, increasing at an accelerating rate as development progressed. In contrast, in diapausing pupae, MR kept decreasing, which suggests that the diapausing phenotype is built upon the least developed pupal state, after the larval tissues have been broken down. Furthermore, the warm diapause treatment demonstrates that the maintenance of diapause in *P. napi* is unaffected by warm temperature, and that diapause termination is dependent on the time spent at low temperature.

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Diapause energetics

One of the main aims of this study was to determine the primary energy store accumulated prior to diapause and its utilization during diapause. The RQ data document a shift from carbohydrate/protein-based energy production to lipid-based energy production in diapause, which persists throughout diapause, suggesting that, as in other insects, diapause in *P. napi* is fueled through lipid metabolism (Hahn and Denlinger, 2007, 2011). Furthermore, lipid analyses suggested that the majority of lipid in 6 day old diapausing pupae (70%) is storage lipid, mainly TAG, which accumulated at a higher proportion in diapausing than in non-diapausing pupae. Therefore, it was surprising that no detectable decrease in storage lipid content occurred as diapause progressed. These data suggest that another energy source is used during winter to fuel metabolism or, alternatively, that lipids are used at a very slow rate. While low RQ values suggest the latter option, they are well below values expected from pure lipid catabolism (Schmidt-Nielsen, 1991). Also, other studies have reported very low RQ values (0.1–0.6) in diapausing insects (Dreyer, 1932; Kleinman, 1934; Agrell, 1951) and have suggested that low temperatures associated with diapause might activate an unknown metabolic shift. Also, in the current study, depression of RQ was related to diapause at low temperature (see Results), and this temperature-dependent depression of RQ might mask fuel use. Several studies have suggested shifts to fuel sources other than lipid catabolism, such as glycolysis, during diapause (Michaud and Denlinger, 2007, Emerson et al., 2010; Ragland et al., 2010). However, these anaerobic processes should raise RQ, rather than lower it. An example of an anabolic alternative that could lower RQ is if carbohydrates are synthesized from free FAs and not funneled into glycolysis but instead retained (e.g. as cryoprotectants) (Storey and Storey, 1991). Nevertheless, while the fuel source determined through RQ could be masked by confounding temperature effects in the cold diapause group, the warm diapause RQ still suggests slow lipid catabolism throughout diapause. However, other fuel sources cannot be ruled out and measuring carbohydrate fluxes during diapause in *P. napi* is an important next step.

Indirectly, observations on insects that can overwinter for multiple years in extended diapause also suggest slow utilization of stored energy (Hanski, 1988; Tauber and Tauber, 2002), especially under optimal conditions, as the stable 2°C used here probably reflects (Williams et al., 2012). Pupae overwintering in the field face both higher day temperatures and larger thermal variability, which is likely to increase energy depletion (Irwin and Lee, 2003). Nonetheless, the warm diapause pupae in the current study showed very little mass loss during the first 4 months in diapause (Fig. 3D), a period that covers the whole endogenous diapause duration (Fig. 2A). As body mass generally is correlated with energy store size (Kono, 1970; Hahn and Denlinger, 2007), energy depletion in *P. napi* during winter is slow. Furthermore, it seems clear that in *P. napi* TAG is saved for post-diapause demands, i.e. post-diapause development, which can be seen by the decrease in TAG content as pupae were moved to a higher temperature and started developing. Indeed, TAG content of 6 day old directly developing pupae was the same as that of 158 day post-diapause pupae (which had spent 7 days at 10°C and 7 days at 20°C).

Interestingly, RQ increased strongly during the post-diapause development period, suggesting a shift towards protein and carbohydrate catabolism during post-diapause development, during the time TAG content decreases most strongly. These findings, however, are not in conflict as in most eukaryotes a carbohydrate deficiency forces the Krebs cycle to provide intermediates for gluconeogenesis, which slows down the cycle and its consumption of acetyl-CoA from β-oxidation of FAs. Thus, active carbohydrate metabolism may promote utilization of TAGs for energy. Previous studies on *P. brassicae* suggest extensive accumulation of sugars in diapausing pupae (Moreau et al., 1982; Pullin and Bale, 1989). If *P. napi* also accumulates sugars during diapause, the shift in RQ suggests these might have dual functions, first as cryoprotectants during diapause and then as fuel for post-diapause development (Storey and Storey, 1991; Ragland et al., 2010).

Lipidome changes during diapause and development

Proximate causes of diapause termination are poorly understood. In the present study we could not detect any shifts in the profiles of lipid classes, individual lipid species or FAs therein during the transition from endogenous diapause to post-diapause quiescence (i.e. comparing the 24 and 114 day points). This suggests that diapause termination is not related to lipid metabolism, but more likely to other physiological processes, such as endocrinological (Jiang et al., 2014; Liu et al., 2015) or sugar metabolism switches (Rubio et al., 2011; Guo et al., 2015). Instead, the largest differences in lipid profiles were seen when comparing direct-developing and diapausing pupae with adults. While there are still relatively few studies investigating lipid dynamics during metamorphosis, our results were in agreement with patterns observed in e.g. *Drosophila melanogaster* (Carvalho et al., 2012) and *Lipoptena cervi* (Mustonen et al., 2015). In our study, CL content was significantly higher in adults than in pupae. As CL is an important structural component of inner mitochondrial membranes (Getz and Bartley, 1959), its content can be seen as a proxy of mitochondrial density, which probably reflects differences in the amount of functional flight muscle tissue (Suarez, 1998). Low numbers of mitochondria in pupae suggest that β-oxidation capacity might be limited during diapause, which supports the notion of slow lipid utilization discussed previously.

Maintaining essential functions of integral proteins of lipid membranes is crucial for surviving diapause, and there are several studies which suggest that acclimatory lipid remodeling is an important part of diapause (Koštál, 2010). Generally, these adjustments increase membrane fluidity, through, for instance, an increase in the degree of membrane unsaturation (Bennett et al., 1997), an increase in PE/PC ratio (Overgaard et al., 2008) or an overall shortening of FAs (Michaud and Denlinger, 2006). However, in the current study no clear signs of cold tolerance or low-temperature functionality-related acclimatory changes were seen, except for an increase in PE/PC ratio when comparing 6 day old diapausing pupae with 24 and 144 day old pupae (from 1.5±0.04 to 1.9±0.07). There could be several reasons for this. When comparing the values in 6 day old diapausing pupae with those of other diapausing insects (Koštál, 2010), even though direct comparisons are challenging, the values in *P. napi* for many traits appear quite high. This could be due to intrinsic developmental processes producing inherently fluid lipid structures. However, as 6 day diapausing and 6 day directly developing pupae differed in all PCA analyses, it seems more likely that 6 day diapausing pupae already show the final diapause phenotype. Another potential explanation is that other forms of protection, such as colligative cryoprotectants (e.g. trehalose) (Pullin and Bale, 1989), buffer membranes to a sufficient degree to uphold functionality. Furthermore, if diapause occurs in a state of early pupal development, large-scale shifts in structural lipid components might not be needed, or at least might not be visible at the whole-

body level, because of poorly developed tissues and organs. However, this final point suggests further analyses should focus on tissue-specific profiles (Carvalho et al., 2012).

Although adults from the direct development pathway eclose at a somewhat less mature stage than adults from the diapause pathway (Karlsson and Johansson, 2008; Larsdotter Mellström et al., 2010), we did not find striking differences in adults between the two pathways regarding any of the physiological parameters measured in this study, except for overall FA U:S ratio, which was higher in post-diapause adults than in directly developing adults (Fig. 6B). The lack of difference suggests canalization of the adult phenotype, probably due to strong selection on adult survival and performance (Waddington, 1942). Membrane phospholipids of adults were in general highly unsaturated and composed of longer acyl chains than those of pupae, as also seen in *L. cervi* (Nieminen et al., 2013; Mustonen et al., 2015). A high degree of unsaturation in membrane lipids has been linked with high MR and efficient transfer of small metabolites through the membrane (Hulbert, 2007). Membrane protein functions are also sensitive to membrane thickness, which is affected by phospholipid chain length (Andersen and Koeppe, 2007). Therefore, elongation of chains when developing from pupae to metabolically highly active adults probably requires adjustment of membrane thickness to be in the range of C16–C18.

Conclusions

Diapause in *P. napi* is an alternative developmental pathway induced by short photoperiods that results in temperature-independent metabolic suppression and maintenance. However, chilling is needed for diapause to progress from endogenous diapause to post-diapause quiescence. During the endogenous phase, diapause cannot be terminated by high temperature and therefore it constitutes a developmental block. After diapause termination, during post-diapause quiescence, development occurs at a very low rate also at low temperature. High temperatures increase development rate, during which post-diapause pupae rapidly assume a developmental trajectory similar to directly developing pupae. Lipid profiles suggest that the diapausing phenotype is built early in development and then persists throughout diapause. Large TAG stores are built and used at a very slow rate during winter, and their primary function appears to be as fuel for post-diapause development. Slow use of lipids might partially relate to a poor capacity of β-oxidation during diapause. Other fuel sources cannot, however, be ruled out. During post-diapause development, proportional TAG content decreases from 70% to 10%. While large changes in the whole-body lipidome were seen between diapausing, developing and adult butterflies at the level of lipid classes, lipid species and fatty acids, no changes in these profiles occurred during diapause maintenance. This indicates that the 6 day old pupae already show all low temperature-related lipid responses needed to survive through diapause development. Instead, stress tolerance (e.g. cold, desiccation)-related acclimation might be more visible in profiles of metabolites (e.g. sugars, polyols, free amino acids) than in lipids. Together, the experiments provide an overview of life history and eco-physiological dynamics in a high latitude population of *P. napi*, and help in understanding the factors driving the evolution of diapause as a life history strategy. The results also highlight the importance of considering diapause and its dynamics when predicting thermal effects of climate change on insects living in seasonal environments.

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Competing interests

The authors declare no competing or financial interests.

Author contributions

P.L., P.P., R.K., S.N., C.W.W., C.W. and K.G. designed the study. C.W. collected butterflies from the field. P.P., M.C., P.L. and K.G. set up the respirometry instrument. P.L., P.P. and D.P. performed the diapause intensity and metabolic rate experiments and R.K. and P.T. performed the lipidomic analyses. P.L. and K.G. did initial data analyses and wrote the first draft of the paper, and all authors contributed to the final version of the paper.

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Data availability

All data associated with this study are available from the Dryad digital repository: http://dx.doi.org/10.5061/dryad.2c2n7 (Lehmann et al., 2016).

Supplementary information

Supplementary information available online at
http://jeb.biologists.org/lookup/doi/10.1242/jeb.142687.supplemental

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