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A comparison of low temperature biology of *Pieris rapae* from Ontario, Canada, and Yakutia, Far Eastern Russia

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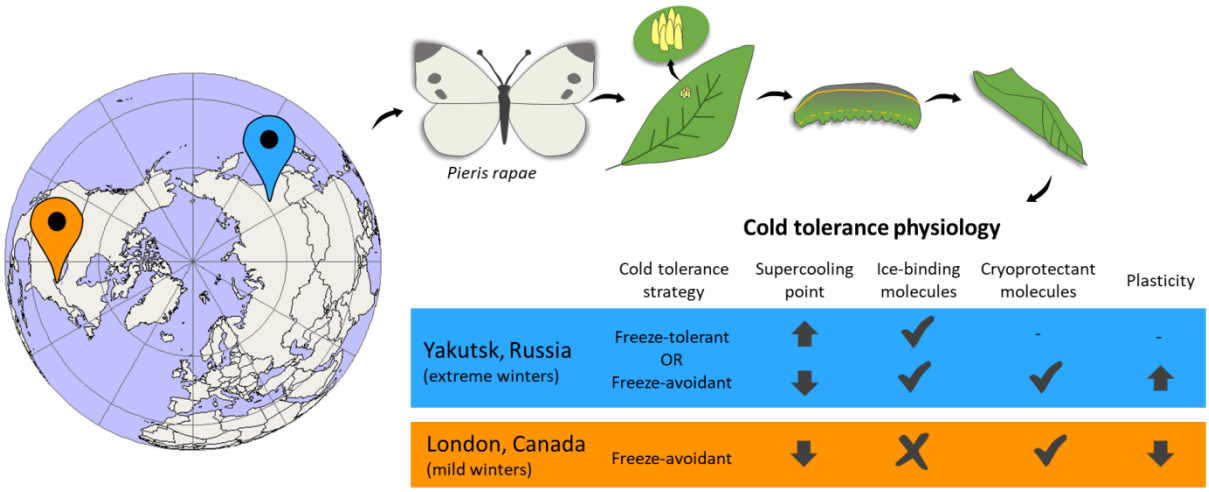
Abstract

Low temperatures limit the distribution and abundance of ectotherms. However, many insects can survive low temperatures by employing one of two cold tolerance strategies: freeze avoidance or freeze tolerance. Very few species can employ both strategies, but those that do provide a rare opportunity to study the mechanisms that differentiate freeze tolerance and freeze avoidance. We showed that overwintering pupae of the cabbage white butterfly *Pieris rapae* can be freeze tolerant or freeze avoidant. A population of *P. rapae* in northeastern Russia (Yakutsk) froze at c. -9.3 °C and were freeze-tolerant in 2002-2003 when overwintered outside. However, *P. rapae* from both Yakutsk and southern Canada (London) acclimated to milder laboratory conditions in 2014 and 2017 froze at lower temperatures (< -20 °C) and were freeze-avoidant. Summer-collected *P. rapae* larvae (collected in Yakutsk in 2016) were partially freeze-tolerant, and decreased the temperature at which they froze in response to starvation at mild low temperatures (4 °C) and repeated partial freezing events. By comparing similarly-acclimated *P. rapae* pupae from both populations, we identified molecules that may facilitate low temperature tolerance, including the hemolymph ice-binding molecules and several potential low molecular weight cryoprotectants. *Pieris rapae* from Yakutsk exhibited high physiological plasticity, accumulating cryoprotectants and almost doubling their hemolymph osmolality when supercooled to -15 °C for two weeks, while London *P. rapae* population exhibited minimal plasticity. We hypothesize that physiological plasticity is an important adaptation to extreme low temperatures (i.e. in Yakutsk) and may facilitate the transition between freeze avoidance and freeze tolerance.

Key words

cryoprotectants, freeze tolerance, freeze avoidance, metabolomics, plasticity

Graphical Abstract



1. Introduction

Low temperatures limit the distribution and abundance of many organisms, especially ectotherms, whose body temperature usually reflects that of the environment (Clarke, 2017). Most insects are chill-susceptible; they die of chilling injury at temperatures well above the temperature at which ice formation begins (supercooling point, SCP; Salt, 1961; Sinclair et al., 2015). Remarkably, many insects survive subzero temperatures by using one of two major cold tolerance strategies: freeze-tolerant insects survive internal ice formation, while freeze-avoidant insects depress the SCP and survive low temperatures as long as no internal ice forms (Lee, 2010; Sinclair et al., 2015). Both freeze avoidance and freeze tolerance are associated with the accumulation of low molecular weight cryoprotectants (e.g. glycerol) and specialized molecules and proteins that help to initiate and control the formation of ice crystals (freeze tolerance) or prevent ice formation and growth (freeze avoidance) (Lee, 2010). Indeed, we have previously hypothesized that freeze tolerance evolved from freeze avoidance based on this accumulation of similar protective molecules (Toxopeus and Sinclair, 2018). However, it is difficult to compare these two cold tolerance strategies without the confounding effects of phylogeny because we lack sufficient phylogenetic resolution for appropriate among-species comparisons, and within-species variation in cold tolerance strategy appears to be the exception rather than the rule.

Cold hardiness can vary substantially within a species, both among and within populations and individuals (Sinclair et al., 2012). Differences in thermal tolerance among populations may result from evolved differences (i.e. fixed genetic changes) or phenotypic plasticity (Mitchell et al., 2011; Schou et al., 2017). Plasticity in cold tolerance can be induced by environmental cues through acclimatization (in the field) or acclimation (in the laboratory) over long periods (days to weeks), and over short periods (minute to hours) in the case of rapid cold hardening (RCH). For example, the spring field cricket *Gryllus veletis* is chill-susceptible during the summer but becomes freeze-tolerant in autumn (acclimatization) or when exposed to decreasing photoperiod and fluctuating temperature in the laboratory (acclimation), a shift in cold tolerance associated with a suite of molecular and physiological changes (Toxopeus et al., 2019a, b). In temperate and polar climates, brief exposures to low temperatures, which can increase cold tolerance, are common during autumn and winter (Marshall and Sinclair, 2012a). For example, the freeze-

tolerant insects *Eurosta solidaginis* (Marshall and Sinclair, 2018) and *Cyphoderris monstrosa* (Toxopeus et al., 2016) accumulate additional cryoprotectants after freezing. Cold tolerance can also vary across development (Jensen et al., 2007); and many insects overwinter in diapause, a state of developmental arrest associated with accumulation of energy reserves, metabolic rate depression, and enhanced stress tolerance (Hahn and Denlinger, 2011).

While inter-individual and inter-population variation in cold tolerance is common, changes in cold tolerance strategy (e.g. switches between freeze tolerance and freeze avoidance) within a species are rare (Addo-Bediako et al., 2000). However, cold tolerance strategy can change over time: overwintering larvae of *Dendroides canadensis* and *Cucujus clavipes* were freeze-tolerant in the late 1970s, and became freeze-avoidant across their entire latitudinal range (35°N to 46°N) in the early 1980s (Kukal and Duman, 1989). Cold tolerance strategy can also change with environment: the linden bug *Pyrrhocoris apterus* and diapausing larvae of the drosophilid *Chymomyza costata* can be freeze avoidant (supercooling to c. -20 °C), but are freeze-tolerant if ice formation is nucleated by external ice at a high sub-zero temperature (> -3 °C) (Rozsypal and Koštál, 2018; Shimada and Riihimaa, 1988). These shifts in cold tolerance strategy can be strongly influenced by environmental cues: *C. costata* acclimated to warm temperatures and long days cannot survive freezing to -32 °C, while their counterparts acclimated to low temperatures and short days can (Koštál et al. 2011). Finally, cold tolerance strategy can vary with geography: overwintering *Acanthocinus aedilis* and *Cossus cossus* appear to survive freezing in the Far East of Russia (Yakutsk) but are freeze-avoidant in northern Europe, where winters are milder (Li, 2016). Thus, natural variation in cold tolerance strategy is perhaps the best opportunity to compare the mechanisms underlying freeze avoidance and freeze tolerance.

The cabbage white butterfly *Pieris rapae* is a potential model to study the mechanisms underlying variation in cold tolerance and cold tolerance strategy (Li and Osakovskii, 2008). This cosmopolitan agricultural pest is native to Europe and North Africa, and has colonized sub-tropical to sub-polar environments across the northern hemisphere (Ryan et al., 2019). *Pieris rapae* is bi- or tri-voltine across much of its distribution and overwinters as a pupa in facultative diapause (Richards, 1940). *Pieris rapae* appears to use more than one cold tolerance strategy

across its geographic range. Overwintering pupae from Estonia (Tallinn, average February temperature = -5.8 °C; en.climate-data.org) freeze at low temperatures (mean SCP = -25.8 °C) and are freeze-avoidant (Sømme, 1982), while those in the Far East of Russia (Yakutia, average February temperature = -35.5 °C; en.climate-data.org) freeze at a temperature much higher than the ambient temperature (mean SCP = -8.5 °C; Li and Averenskii, 2007). Li and Averenskii (2007) used respirometry to demonstrate that *P. rapae* pupae survived freezing, similar to other Siberian insects.

Here, we compare cold tolerance strategy of *P. rapae* larvae and pupae from regions with relatively extreme (Yakutsk, Russia) and mild (London, Canada) winters, using field and laboratory measurements over a period of 15 years. To identify mechanisms that might support switches in cold tolerance strategy, we compare cold tolerance and hemolymph composition (and plasticity thereof) of Yakutsk and London pupae acclimated to common laboratory conditions. While not all of our experiments were synchronized, our results suggest that *P. rapae* from Yakutsk have highly plastic physiology, and that their cold tolerance strategy is not fixed.

2. Material and methods

2.1 Insect collection, rearing, acclimatization, and acclimation

We collected *P. rapae* in the late summer (August or September) of 2002, 2014, 2016, and 2017 from Yakutsk, Russia (62.0°N, 129.7°E) and/or London, Canada (43.0°N, 81.3°W), and acclimated or acclimatized them as summarized in Table 1. The average minimum temperatures (2019) in the coldest winter month (January) of these two collection locations are -44.7 °C and -10.0 °C, respectively. Both populations are invasive (Ryan et al., 2019), but likely experience limited gene flow due to their geographical separation.

Table1. Collection and treatment of *Pieris rapae* populations used in this study. *

Collection year	Populations (*N*)	Stage	Acclimation/acclimatization	Experiments
2002	Yakutsk (18)	Pupa	Acclimatization to local overwintering conditions (Oct 2002 to April 2003)	Seasonal SCP and CTS measurements
2014	Yakutsk (13), London (2)	Pupa	Acclimation to decreasing fluctuating temperatures and photoperiod (12 weeks)	SCP and CTS measurements following acclimation
2016	Yakutsk (16)	Larva	Acclimation to room temperature for up to 1 week	SCP and CTS measurements following acclimation, starvation, and repeated freezing
2017	Yakutsk (7), London (6)	Pupa	Acclimation to 4 °C (12 weeks), ± supercooling to -15 °C for weeks	SCP and CTS measurements; hemolymph composition following acclimation and supercooling

*CTS, cold tolerance strategy; *N*, samples size for experiments; SCP, supercooling point;

We collected Yakutsk *P. rapae* as late (third) instar larvae (caterpillars) from cabbage fields in all four years of the study. We maintained larvae at room temperature (c. 22 °C) under natural light conditions with daily access to fresh organic cabbage until pupation (2002, 2014, 2017) or until use in cold tolerance experiments (2016). In 2002, we transferred individuals to 4 °C (total darkness) within two days of pupation (late August) and then transferred them to an outdoor, snow-covered field cage (also in total darkness) from October 2002 until April 2003 in Yakutsk. Groups of two or three pupae were transferred back to the laboratory each month for cold tolerance experiments. In 2014 and 2017, we transported pupae in individual containers (1.7 ml microcentrifuge tubes with a punctured lid) to the University of Western Ontario (London, Canada) on ice for two days (2014) or at room temperature for two weeks (2017). We acclimated these *P. rapae* to the same laboratory conditions as the pupae collected in London (described below) prior to use in cold tolerance experiments.

We collected London *P. rapae* as adults from an urban garden in August and September in 2014 and 2017. We maintained adults in a 61 × 61 × 61 cm tent-shaped bug dorm (BioQuip, Compton, CA, USA) at room temperature (c. 22 °C) under natural light conditions with fresh organic

cabbage for oviposition and artificial nectar (10 % honey solution) replenished three times per week. We reared offspring of these adults under the same conditions. In 2014, individuals that pupated by mid-September were transferred (along with Yakutsk pupae) to an acclimation with decreasing, fluctuating temperatures and photoperiod for 12 weeks (until early December) in a Sanyo MIR 154 incubator (Sanyo Scientific, Bensenville, IL, USA) prior to use in cold tolerance experiments. Temperatures and photoperiod conditions were selected to approximately mimic autumn conditions in London, Canada (Table 2). In 2017, pupae were kept at room temperature for 2 weeks and then transferred along with Yakutsk pupae to 4 °C (total darkness) for 12 weeks prior to use in experiments. Pupae that were not in diapause (i.e. eclosed during those two weeks at temperature) or that were parasitized were excluded from cold tolerance and plasticity experiments (described below).

Table 2. Temperature and photoperiod acclimation conditions over 12 weeks used for *P. rapae* pupae collected in 2014. Each condition was used for 2 weeks (14 days). Pupae were exposed to the high and low temperatures for 12 h each.

Weeks	Temperature (°C)		Photoperiod (L:D, h)
	High	Low	
1 and 2	17	15	11:13
3 and 4	15	10	11:13
5 and 6	15	8	9:15
7 and 8	12	6	9:15
9 and 10	8	4	8:16
11 and 12	4	2	8:16

2.2 Cold tolerance experiments

To determine the SCP of individuals from all four collection years, we cooled *P. rapae* pupae and larvae at a rate of 1 °C min^{-1} from room temperature to the SCP. Each individual was cooled in its own container (e.g. 1.7 ml microcentrifuge tube), in contact with a Type T copper-constantan thermocouple (Omega Engineering, Laval, QC, Canada) to continuously measure temperature, either through connection to a INSEIS L 120 E Line Recorder (2002-2003, 2016; Wavefield Inseis, Bergen, Germany) or PicoLog TC-08 unit interfaced to a computer with PicoLog v5.24.1 software (2014, 2017; Pico Technology, Cambridge, UK). Each container was cooled in either a TC-G-180 Binder climatic chamber (2002-2003, 2016; Tuttlingen, Germany)

or an aluminum block connected to a Lauda Proline 3530 recirculating bath containing methanol (2014, 2017; Lauda, Würzburg, Germany). The SCP was defined at the lowest temperature observed prior to exotherm formation (increase in temperature associated with the exothermic process of ice formation; Sinclair et al., 2015). We compared SCP among groups using ANOVA with a Tukey's post-hoc test. All statistical analyses were conducted in R version 3.2.2 (R-Core-Team, 2019).

We determined the cold tolerance strategy of *P. rapae* in all four collection years. Following the acclimation or acclimatization described in Table 1, we cooled pupae as described above to either the SCP or a temperature just above the SCP, immediately transferred them to room temperature and assessed survival (Sinclair et al., 2015). The methods to determine survival included: respirometry of pupae one day post-thaw (2002-2003; details below), eclosion of pupae as adults (2014), and both respirometry of pupae five days post-cold and eclosion as adults (2017). We classified populations as freeze-tolerant if individuals survived following a completed exotherm and freeze avoidant if only unfrozen individuals survived (Sinclair et al., 2015). In 2017, for a subset of pupae, we inoculated freezing at a high sub-zero temperature (c. -4 °C) using external application of a silver iodide slurry (cf. Toxopeus et al., 2019b).

To determine larval cold tolerance strategy, we cooled field-collected third instar larvae (2016) to their SCP and compared survival of larvae returned to room temperature soon after either full or partial exotherm formation. We defined survival as the ability of larvae to move without stimulus or prompting after three days at room temperature. We classified populations as partially freeze-tolerant if individuals survived following partial, but not full, exotherm formation (Sinclair, 1999). To investigate plasticity in larval cold tolerance, we measured the SCP of larvae that were actively feeding at room temperature, and larvae that were starved for one or five days at 4 °C. Because we determined that larvae could survive partial freezing, we tested whether repeated short freeze treatments (such as might occur during autumn cold snaps) impacted larval SCP and survival. These larvae were cooled to their SCP, immediately returned to room temperature prior to exotherm completion, and held at room temperature for c. 30

minutes between freeze treatments. We used a linear regression to determine if SCP changed as the number of freeze treatments increased.

2.3 Respirometry

We measured CO_2 emission by cold-exposed pupae to determine survival in 2002-2003 and 2017. All measurements for survival were conducted at 20 °C for expediency; diapause pupae exhibit a long (> 6 h) discontinuous gas exchange cycle at lower temperatures (e.g. 10 °C). In 2002-2003, we conducted respirometry using an Engelmann constant pressure respirometer as described previously (Zachariassen et al., 1987). In 2017, we used Sable Systems flow-through respirometry (Sable Systems International, Las Vegas) as described previously (Toxopeus et al., 2019b). For flow-through respirometry, we corrected CO_2 production to 5 min baseline measurements and calculated the rate of CO_2 production ($\dot{V}CO_2$; Lighton, 2018). Pupae with a mass-specific $\dot{V}CO_2$ greater than 40 µl CO_2 g^{-1} h^{-1} were classified as alive. In 2017, five of the six pupae (London and Yakutsk populations) that we classified as 'alive' based on respirometry also eclosed as adults, indicating that respirometry is a reliable metric of survival in diapausing *P. rapae*. We also conducted respirometry on a small number of diapause and non-diapause pupae from the 2017 London population to characterize typical gas exchange patterns at 10 °C and 20 °C. We classified pupae as non-diapause if they eclosed within two weeks of pupation at room temperature, while diapause pupae had no apparent development during this time. We compared CO_2 production of diapause and non-diapause pupae at both temperatures using ANCOVA with mass as a covariate.

2.4 Plasticity of hemolymph composition

We measured hemolymph osmolality, thermal hysteresis, and metabolite composition of Yakutsk and London pupae collected in 2017. To determine baseline hemolymph composition, we extracted hemolymph from pupae that were acclimated as described above (2-3 weeks at room temperature followed by 12 weeks at 4 °C). To determine whether hemolymph composition was plastic, we extracted hemolymph from pupae that were acclimated in the same way, then supercooled at -15 °C for two weeks, and returned to 4 °C for five days. We kept these pupae at

4 °C for five days to allow them time to recover and potentially synthesize cryoprotectants post-supercooling. All pupae survived this supercooling treatment, as determined by respirometry at 20 °C. Following respirometry, we extracted up to 40 µl of hemolymph from each individual by making a small posterior incision and pipetting out the liquid using a 10 µl micropipette. Hemolymph samples were flash-frozen in liquid nitrogen and stored at -80 °C until analysis.

We measured hemolymph osmolality using a nanolitre osmometer (Otago Osmometers, Dunedin, New Zealand), as described previously (Toxopeus et al., 2019b). Briefly, we used the osmometer to rapidly freeze small volumes of hemolymph, determined osmolality from the melting point of the solution, and calculated thermal hysteresis from the difference between melting and freezing points (Crosthwaite et al., 2011). We also noted ice crystal shape (hexagonal or spicular) during this process to identify the presence of potential ice-binding molecules. We compared the osmolality of Yakutsk and London pupae before and after supercooling using a two-way ANOVA.

To compare the hemolymph composition among pupae, we quantified low molecular weight metabolites *via* targeted metabolomics in 10 µl samples of hemolymph, as previously described (Rozsypal et al., 2018; Toxopeus et al., 2019a). Briefly, we lyophilized hemolymph samples, and sent them to the Laboratory of Analytical Biochemistry at the Czech Academy of Sciences for metabolomic analysis. The samples were rehydrated and homogenized in 400µl of methanol: acetonitrile: water mixture (2:2:1, v/v/v) containing internal standards (p-fluoro-DL-phenylalanine, methylα-D-glucopyranoside; both at a final concentration of 200 nmol/ml; Sigma-Aldrich). The TissueLyser LT (Qiagen, Hilden, Germany) was set to 50 Hz for 5 min (with a rotor pre-chilled to−20°C). Homogenization was repeated twice and the two supernatants from centrifugation at 20 000 × g for 5 min at 4 °C were combined. To quantify acidic metabolites (e.g. amino acids), samples were derivatized by ethylchloroformate in pyridine/ethanol, and extracted in chloroform (for GC-MS) or 30 % methanol (for LC-MS). To quantify sugars and polyols, samples were derivatized by oximation and methylsilylation, and dissolved in iso-octane for analysis by GC-FID. Each metabolite was quantified (nmol/µl hemolymph) by comparison to a standard curve generated for that metabolite. We conducted

principal component analysis (PCA) using the *calibrate* package in R to compare metabolite profiles of individuals from different treatments and populations. To do so, we first standardized metabolite concentrations by subtracting each concentration from the mean and dividing by the standard deviation of that metabolite. We identified metabolites of interest as those that loaded strongly onto principal components (PCs) 1 and 2. We also determined whether individual metabolites of interest differed in concentration with treatment or population using two-way ANOVAs.

3. Results

3.1 Cold tolerance strategies of P. rapae *across time, population, and treatment*

In 2002-2003, diapausing pupae of *P. rapae* overwintering outside in Yakutsk froze at moderately high sub-zero temperatures (-7.3 to -12.2 °C) and (because all individuals that froze recovered) we classified this population as freeze tolerant (Table 3). Pupal SCP in this winter-acclimatized group was seasonally plastic (Fig. 1A): the highest SCP values were recorded in the autumn (November) and spring (April), and the lowest SCP values in mid-winter (January). Conversely, *P. rapae* pupae from Yakutsk that were collected in the late summer of 2014 or 2017 and acclimated to laboratory conditions in London, Canada froze at between -20.6 and -26.5 °C, and did not survive freezing, but did survive cooling to temperatures above the SCP (Table 3). We classified these populations as freeze avoidant. Pupae from London that were collected and acclimated in 2014 or 2017 were similarly freeze-avoidant and froze between -20.9 and -24.7 °C (Table 3). No pupae collected in 2017 survived freezing when ice formation was nucleated with silver iodide.

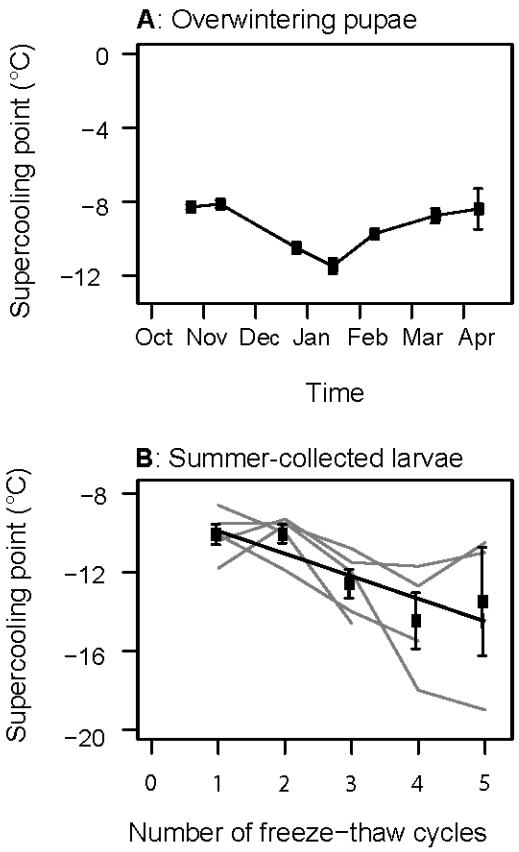
We classified *P. rapae* larvae collected from Yakutsk in late summer (August) of 2016 as partially freeze tolerant: they froze at moderate sub-zero temperatures (-8.8 to -13 °C) and they survived ice formation associated with partial exotherm completion, but did not survive if exotherm formation completed (Table 3). Larval SCP was plastic: if larvae were partially frozen several times in a row, SCP tended to decrease with each subsequent freeze treatments, reaching

values as low as -19 °C (Fig. 1B). When starved and kept at 4 °C for five days, larval SCP decreased by c. 1 °C, in concert with mass loss (c. 25 %) and clearing of gut contents (Table S1).

Table 3. Mean supercooling points (SCPs) and cold tolerance strategy of *Pieris rapae* third instar larvae and diapausing pupae. Treatments of individuals prior to freezing or supercooling are summarized in Table 1. The fraction of individuals that survived freezing, partial freezing, or supercooling are indicated for each group.

Collection year	Population	Stage	Mean SCP ± s.e.m. (°C)*	*N* survived/ *N* frozen	*N* survived/ *N* partially frozen	*N* survived/ *N* super-cooled	Cold tolerance strategy
2002	Yakutsk	Pupa	-9.3 ± 0.3[a]	18/18	n.d.	n.d.	Freeze tolerant
2014	London	Pupa	-22.6 ± 1.7[b]	0/2	n.d.	n.d.	Cannot discern
	Yakutsk	Pupa	-23.6 ± 0.7[b]	0/9	n.d.	2/4	Freeze avoidant
2016	Yakutsk	Larva	-10.2 ± 0.5[a]	0/8	8/8	n.d.	Partially freeze tolerant
2017	London	Pupa	-24.4 ± 0.3[b]	0/3	n.d.	3/3	Freeze avoidant
	Yakutsk	Pupa	-20.8 ± 0.8[b]	0/2	n.d.	3/5	Freeze avoidant

N, sample size; n.d., no data; s.e.m. standard error of the mean
*Differences in mean SCP among groups are indicated by different letters (ANOVA, Tukey's post-hoc test, $P < 0.05$).



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309 **Figure 1**. Mean SCP of *Pieris rapae* **(A)** pupae overwintering in Yakutsk from October 2002 to
310 April 2003 and **(B)** larvae collected in 2016 and partially frozen up to five consecutive times.
311 Overwintering pupae were kept in darkness in a snow-covered overwintering field cage, and two
312 or three individuals were removed each month for SCP measurements. Larvae were cooled to
313 their SCP, returned to room temperature prior to exotherm completion, and held at room
314 temperature for 30 min between freeze treatments. Mean ± s.e.m. is displayed for each time point
315 or freeze treatment; solid black line indicates the linear regression. Data from each larva are
316 represented by grey lines, and mean SCP decreased as the number of freeze-thaw cycles
317 increased ($F_{20,1}$ = 10.77, P = 0.004). Small error bars are obscured by symbols.

3.2 Respirometry

Diapausing pupae from the London population (2017) exhibited discontinuous gas exchange at both 10 °C and 20 °C, while non-diapause pupae exhibited continuous gas exchange at 20 °C and discontinuous gas exchange at 10 °C (example respirometry traces in Fig. 2A-D). Non-diapause pupae had higher metabolic rates than diapause pupae at both temperatures (Fig. 2E). We show an example respirometry trace from a dead pupa in Fig. 2F.

3.3 Plasticity of hemolymph composition

Pieris rapae pupae had hemolymph osmolality ranging from 355 to 505 mOsm when acclimated to room temperature for two weeks followed by 4 °C for 12 weeks (2017). The hemolymph osmolality of London pupae was c. 115 mOsm higher than that of Yakutsk pupae following this acclimation (Fig. 3). When acclimated pupae were subsequently supercooled to -15 °C for two weeks and returned to 4 °C for five days, hemolymph osmolality of Yakutsk pupae almost doubled, while that of London pupae did not change compared to pupae that were only held at 4 °C (Fig. 3). We detected negligible thermal hysteresis (< 0.05 °C) in all hemolymph samples. However, most frozen hemolymph samples from Yakutsk pupae contained hexagonal or spicular ice crystals, suggesting *P. rapae* have hemolymph ice-binding proteins (Table S2). Ice crystals in hemolymph from London were circular, suggesting a lack of ice-binding molecules.

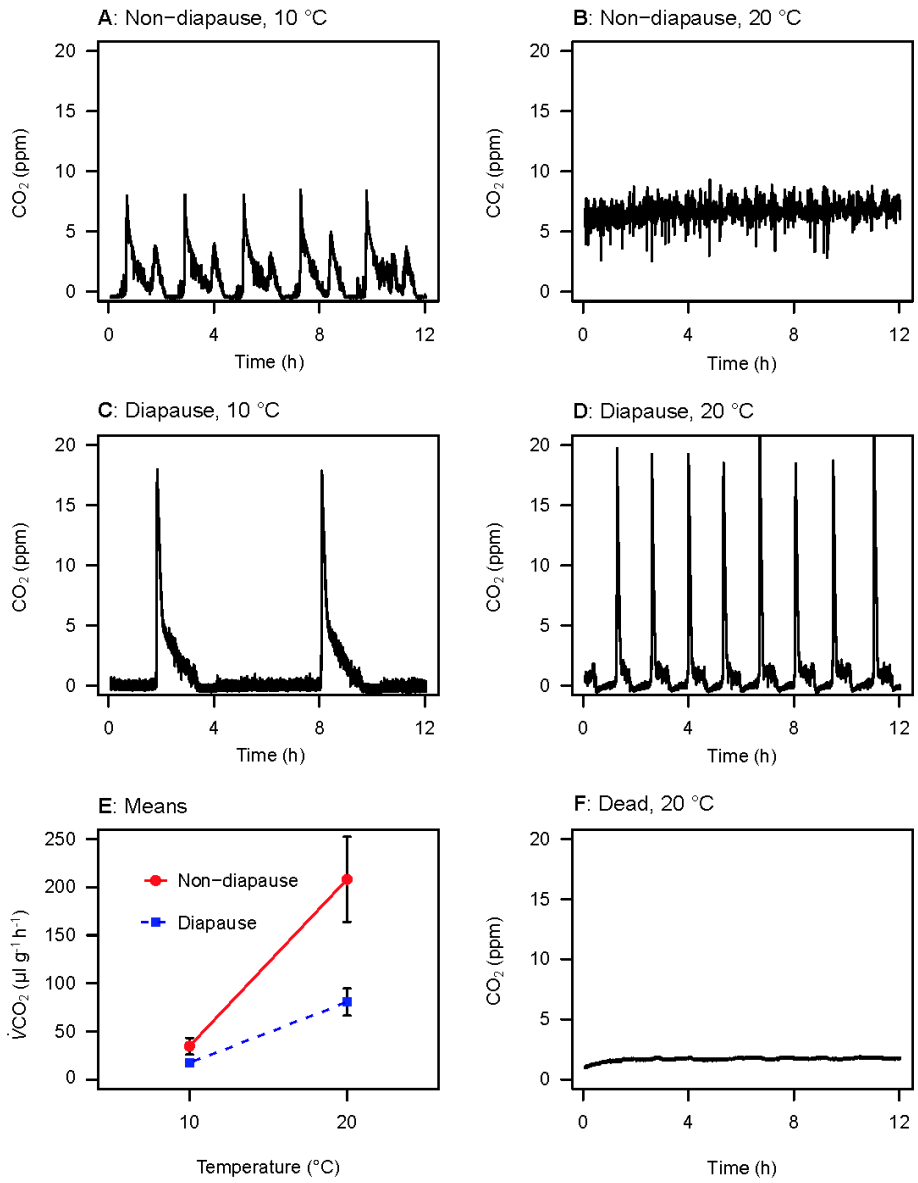


Figure 2. Example respirometry traces of **(A,B)** non-diapause, **(C, D)** diapause *Pieris rapae* pupae from London in 2017. **(E)** Mean (± s.e.m.) rates of CO_2 production ($\dot{V}CO_2$) for three diapause and non-diapause pupae from the same population. Diapause and temperature impacted mean rates of CO_2 production (ANCOVA, Diapause status: $F_{12,1} = 9.77$, $P = 0.017$; Temperature: $F_{12,1} = 26.52$, $P = 0.001$; Diapause status × Temperature: $F_{12,1} = 5.71$, $P = 0.048$). **(F)** Example respirometry trace of a dead *P. rapae* pupa. CO_2 production was measured over a 12 h period at 10 °C or 20 °C.

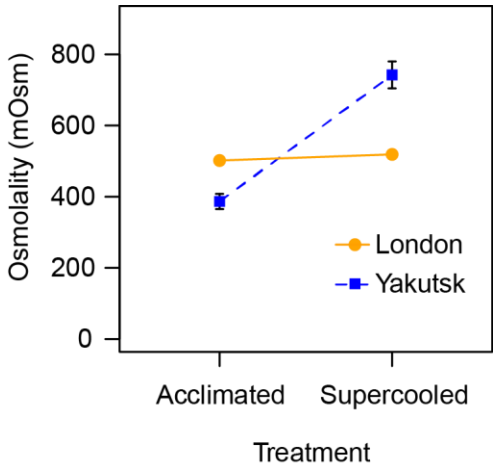
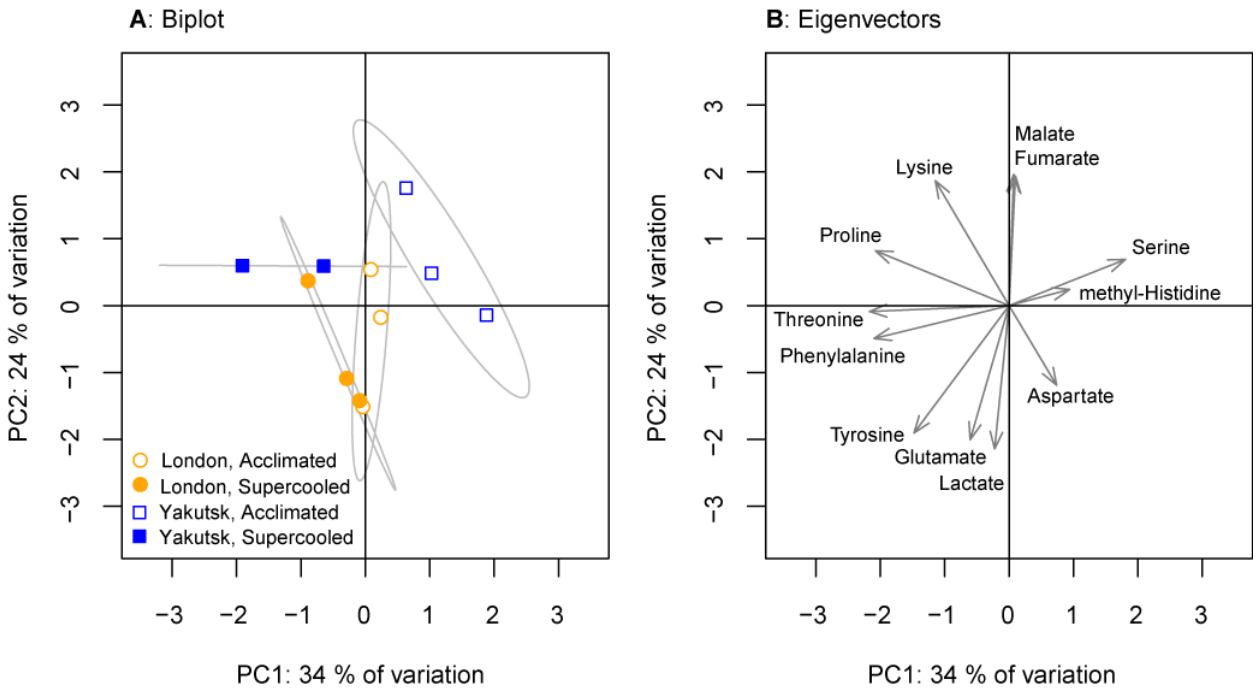


Figure 3. Hemolymph osmolality (mOsm) of *Pieris rapae* diapause pupae. London and Yakutsk pupae in 2017 were acclimated at 4 °C for 12 weeks ('acclimated'), and a subset were then cooled to -15 °C for two weeks ('supercooled') and returned to 4 °C for five days prior to osmolality measurement. Mean ± s.e.m. of three pupae is displayed for each population × treatment combination. Small error bars are obscured by symbols. Supercooling increased osmolality of Yakutsk pupae (ANOVA, Population: $F_{11,1} = 3.32$, $P = 0.11$; Treatment: $F_{11,1} = 72.41$, $P < 0.001$; Population × Treatment: $F_{11,1} = 75.33$, $P < 0.001$).

We quantified 46 hemolymph low molecular weight metabolites from these same pupae and analyzed the impact of supercooling on metabolite composition. The metabolite profile of Yakutsk pupae changed substantially with supercooling, whereas we saw little shift in the hemolymph composition of London pupae (Fig. 4A). Most of the variation in Yakutsk hemolymph composition was associated with metabolites along PC 1, including amino acids (alanine, isoleucine, leucine, phenylalanine, proline, serine, threonine), polyols (sorbitol), and citric acid cycle intermediates (aconitic acid, ketoglutaric acid, citric acid) (Fig. 4B). Approximately 220 mOsm of the c. 350 mOsm increase in Yakutsk pupa osmolality could be accounted for by the accumulation of low molecular weight metabolites, many of which are potential cryoprotective molecules. The metabolites with moderate increases (> 20 mM) in concentration following supercooling in the Yakutsk population were proline, alanine, glutamine, sorbitol, and fructose (Fig. 5). Small increases (> 5 mM) were observed in leucine, isoleucine, threonine, glycerol, mannitol, and trehalose (Fig. 5).



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369 **Figure 4**. Principal component analysis (PCA) of hemolymph metabolites in London and
370 Yakutsk pupae collected and acclimated in 2017. Pupae were acclimated at 4 °C for 12 weeks
371 ('acclimated'), and a subset were then cooled to -15 °C for two weeks ('supercooled') and
372 returned to 4 °C for five days prior to quantification of metabolites. **(A)** Biplot of the PCA, with
373 each pupal hemolymph sample represented by a point; the 95 % confidence interval is
374 represented by an ellipse. **(B)** Eigenvectors of the 12 metabolites that loaded most strongly onto
375 PCs 1 and 2; longer arrows indicate a greater impact on the PC.
376

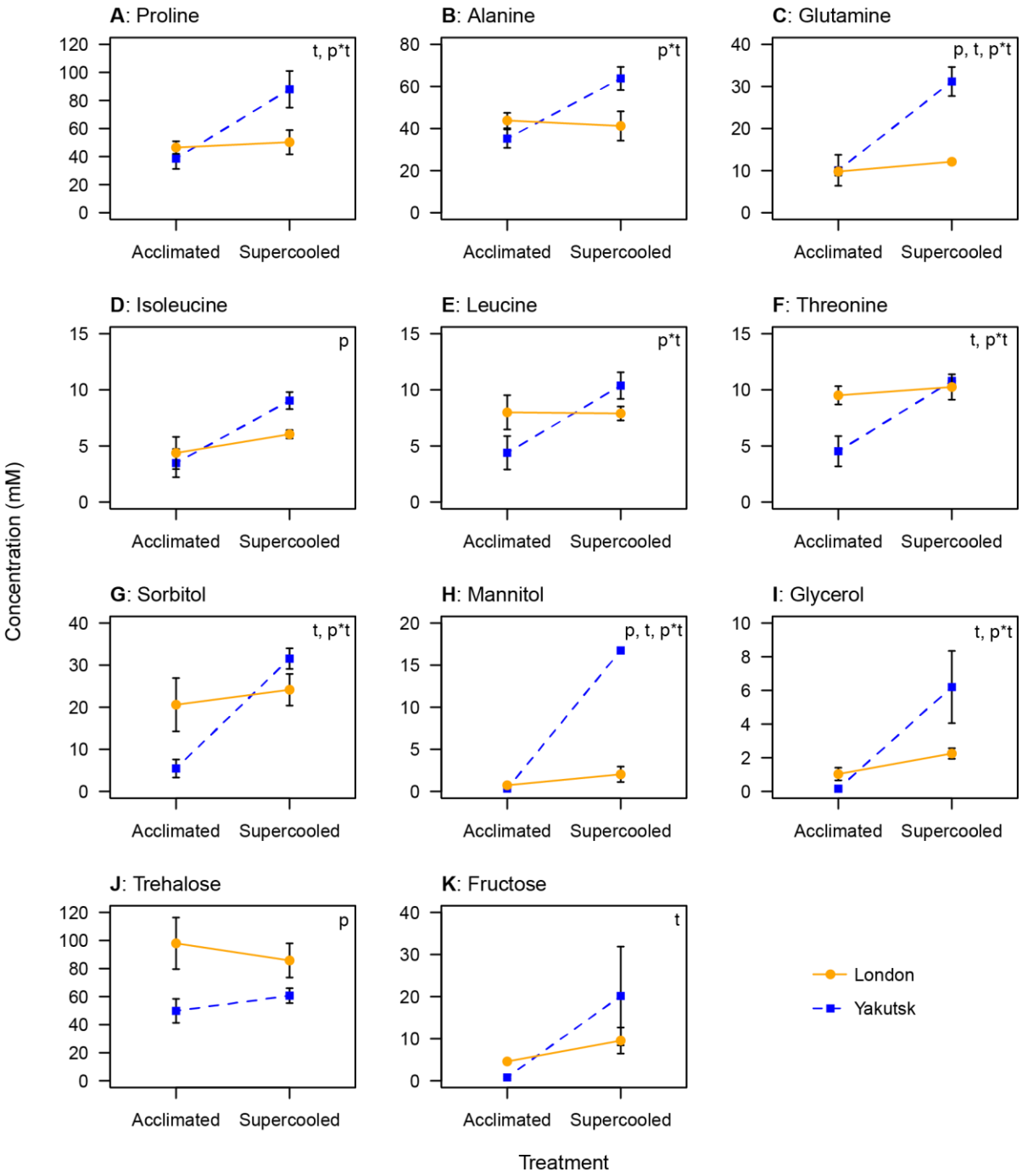

Figure 5. Hemolymph concentrations (mM) of **(A-F)** amino acids, **(G-I)** polyols, and **(J-K)** sugars with moderate to substantial changes in concentration following supercooling. London (orange) and Yakutsk (blue) pupae in 2017 were kept at 4 °C for 12 weeks ('acclimated'), and a subset were then cooled to -15 °C for two weeks ('supercooled') and returned to 4 °C for five days prior to quantification of metabolites. Mean ± s.e.m. of three pupae is displayed for each Population × Treatment combination. Small error bars are obscured by symbols. Significant effects of Population (p), Treatment (t), or Population × Treatment (p*t) interaction on cryoprotectant concentration are indicated in each panel (ANOVA, $P < 0.05$).

4. Discussion

4.1 The cold tolerance strategy of Pieris rapae *varies with population, time, and acclimation*

Overwintering *P. rapae* pupae can be either freeze tolerant or freeze avoidant. Pupae overwintering outdoors in Yakutsk, Russia in 2002-2003 were freeze tolerant (see also Li and Averenskii, 2007), whereas diapausing *P. rapae* from the milder climate of London, Canada were freeze avoidant (cf. Estonian populations; Sømme, 1982). Although field-collected Yakutsk pupae in 2002-2003 were freeze tolerant, Yakutsk pupae acclimated to laboratory conditions in 2014 and 2017 were freeze avoidant. It should be noted that we had low sample sizes for determining cold tolerance strategy; however, cold tolerance strategy is binary in cold-tolerant species (Sinclair et al. 2015). There was limited variability in survival results (e.g. all 2002-2003 Yakutsk pupae survived freezing, whereas no pupae survived freezing in 2014 or 2017), and we are therefore confident in these results. We propose two hypotheses for the shift we observed in cold tolerance strategy: 1) the *P. rapae* population in Yakutsk has lost its ability to become freeze-tolerant over time; or 2) the laboratory acclimation conditions we used in 2014 and 2017 were insufficient to induce freeze tolerance.

There is precedent for hypothesis 1 (loss of freeze tolerance over time) in the beetles *D. canadensis* and *C. clavipes* (Kukal and Duman, 1989). Both of these species were freeze-tolerant when first studied, but individuals collected from the same location in subsequent years were freeze avoidant, apparently due to the loss of ice nucleating agents (Kukal and Duman, 1989). We saw a loss of freeze tolerance in *P. rapae* pupae collected from Yakutsk between 2002 and 2014, concurrent with winter warming: air temperatures were below -50 °C in 2002-2003, but rarely dropped below -40 °C in 2014-2015 (en.climate.org). However, we cannot truly assess hypothesis 1 in this population because the freeze-tolerant pupae we collected in 2002-2003 were treated very differently (overwintered outdoors) from the freeze-avoidant pupae we studied in 2014 and 2017 (laboratory-acclimated to mild conditions).

We favour hypothesis 2 (that we could not artificially induce freeze tolerance in Yakutsk pupae) because we expect that freeze avoidant pupae would not be able to suppress their SCP

sufficiently to remain unfrozen during a Yakutian winter. *Pieris rapae* pupae overwinter attached to the leaves and stems of Brassica plants (e.g. cabbage), and may be directly exposed to the winter air temperatures unless winter precipitation is substantial. Even though snow cover could provide some insulation, winter air temperatures below -40 °C (en.climate-data.org) would likely making it very difficult for *P. rapae* to avoid internal ice formation (Ring, 1982). We therefore expect freeze tolerance to be a necessary cold adaptation mechanism for Yakutsk *P. rapae*, similar to other overwintering insects in regions with extreme low temperatures (Li, 2016; Toxopeus and Sinclair, 2018). Conversely, freeze avoidance is likely sufficient for *P. rapae* to overwinter in milder climates. In London, minimum winter air temperatures are rarely below -24 °C, and there is significant buffering by snow cover (Marshall and Sinclair, 2012b), which means that *P. rapae* likely remain unfrozen (and alive) during winter.

We suggest that the laboratory acclimation conditions in 2014 and 2017 lacked critical environmental cues that induce physiological changes required for freeze tolerance in Yakutsk *P. rapae*. For comparison, *G. veletis* requires a complex acclimation regime to induce freeze tolerance, including decreasing day length combined with decreasing fluctuating temperatures over six weeks (Toxopeus et al., 2019b). If either day length or temperature cues are missing, *G. veletis* does not become freeze tolerant (Toxopeus et al., 2019b). The autumn temperatures in Yakutsk are considerably lower and the days are much shorter than those in London, so our laboratory acclimations (e.g. Table 2) likely lacked the multiple cues required to induce freeze tolerance. Thus, although the *P. rapae* we collected in 2014 and 2017 were freeze-avoidant, we expect that *P. rapae* collected in Yakutsk and acclimated to laboratory conditions that closely mimic environmental temperature and photoperiod of autumn in Yakutsk will be freeze tolerant.

4.2 Yakutsk populations exhibit greater plasticity in cold tolerance physiology than London populations

We observed little inter-population variation in whole organism cold tolerance metrics when *P. rapae* pupae were acclimated to laboratory conditions (4 °C for 12 weeks) in 2017. Both Yakutsk and London populations were freeze avoidant, froze at similar temperatures (mean

SCPs of -20.8 °C and -24.4 °C, respectively), and all pupae tested were able to survive at -15 °C for two weeks. Unlike in *P. apterus* and *C. costata* (Rozsypal and Koštál, 2018; Shimada and Riihimaa, 1988), external nucleation of ice formation at a high sub-zero temperature did not induce freeze tolerance. However, when we supercooled (2 weeks at -15 °C) acclimated Yakutsk and London pupae (2017), we detected inter-population differences at the suborganismal level. We observed substantial plasticity in hemolymph composition (increased osmolality and metabolite concentrations) of Yakutsk *P. rapae* in response to supercooling, unlike their London counterparts. Although dehydration alone can increase hemolymph osmolality (e.g. Bennett et al., 2005), our metabolomics results suggest that Yakutsk pupae altered hemolymph osmolality through cryoprotectant synthesis. We did not see a uniform increase in metabolite concentrations (which we would expect with dehydration) in supercooled Yakutsk pupae: glycerol and mannitol increased by more than 30-fold, while proline concentrations merely doubled (Fig. 5), and serine and succinic acid concentrations decreased (see supplementary data). Synthesis of cryoprotective metabolites can underlie substantial increases in cold tolerance, both over short (e.g. < 6 h in *B. antarctica*) and long (e.g. 6 weeks in *G. veletis*) time scales, even when metabolic rates are suppressed (Lee et al., 2006; Michaud et al., 2008; Toxopeus et al., 2019a). This plasticity may also be important for switches in cold tolerance strategy; for example, *E. solidaginis* from low latitudes (29°N) depress their SCP (avoid freezing) in late autumn, but accumulate additional cryoprotectants (glycerol) and become freeze-tolerant in mid-winter (once environmental temperatures decrease below this SCP; Baust and Lee, 1981). Thus, although we were unable to compare freeze tolerance and freeze avoidance in a common-garden approach, our results suggest a role for plasticity in cold tolerance of the more cold-adapted (Yakutsk) population of *P. rapae*.

4.3 Mechanisms facilitating cold tolerance of Pieris rapae

Similar to many insects, cold tolerance varied with development stage in *P. rapae*. Summer-collected *P. rapae* larvae from Yakutsk (2016) were partially freeze-tolerant. Early autumn temperatures in Yakutsk can drop below 0 °C (en.climate-data.org), and larval partial freeze tolerance may facilitate survival of sub-zero temperatures prior to pupation. Larvae decreased their SCP in response to starvation, similar to freeze-avoidant insects that clear their gut to

remove exogenous ice nucleators (e.g. Olsen and Duman, 1997). Larval SCP also decreased following multiple freezing events, similar to repeatedly-frozen larvae of *Syrphus ribesii* (Brown et al., 2004). The mechanism underlying this response to repeated freezing is unknown, and we speculate that dehydration (loss of freezable water) or rapid cryoprotectant synthesis between freezing events could cause the SCP depression we observed over time. Cold tolerance increased with pupation and entry into diapause, as pupae became freeze-tolerant (2002-2003) or freeze-avoidant with low SCPs (2014, 2017). Diapause pupae had lower metabolic rates than non-diapause pupae, consistent with many insects (Hahn and Denlinger, 2011), likely facilitating overwintering survival by conserving energy reserves (Sinclair, 2015).

In addition to developmental and metabolic changes, we identified biochemical changes that could facilitate *P. rapae* cold tolerance. Most cold-hardy insects accumulate low molecular weight cryoprotectants (Lee, 2010; Toxopeus and Sinclair, 2018), and even moderate (e.g. c. 20 mM) increases in concentrations of these low molecular weight metabolites can enhance cold tolerance, both in chill-susceptible (e.g. Koštál et al., 2012) and cold-tolerant species (e.g. Toxopeus et al., 2019a). Supercooled Yakutsk *P. rapae* pupae accumulated potential cryoprotectants similar to other freeze-tolerant insects, including c. 85 mM proline (cf. Koštál et al., 2011; Toxopeus et al., 2019a), c. 60 mM alanine (cf. Michaud et al., 2008), and c. 30 mM sorbitol (cf. Baust and Lee, 1981). Proline has a demonstrated *in vivo* role in insect freeze tolerance (Koštál et al., 2016; Toxopeus et al., 2019a), and its accumulation likely enhances cold tolerance in *P. rapae*. We measured hemolymph [trehalose] > 50 mM in both London and Yakutsk *P. rapae* pupae, within the range of concentrations seen in freeze-avoidant and freeze-tolerant species (Purać et al., 2016). This common cryoprotectant may facilitate cold tolerance in both *P. rapae* populations.

In addition to low molecular weight cryoprotectants, we investigated inter-population differences in hemolymph ice-binding agents. Freeze-tolerant insects often produce hemolymph ice-nucleating agents (INAs) that initiate ice formation at high sub-zero temperatures (Toxopeus and Sinclair, 2018), as previously detected in lepidopterans from Yakutsk (Li, 2012). We did not see the elevated SCPs indicative of hemolymph INAs in pupae from either London or Yakutsk in

2014 or 2017, and speculate that our acclimation conditions were not appropriate to induce INA accumulation (although we did not directly measure INA activity). However, we detected spicular or hexagonal ice crystals in most Yakutsk (but few London) hemolymph samples (2017), suggesting the presence of other ice-binding agents. All of the hemolymph samples we tested had minimal thermal hysteresis (< 0.05 °C), suggesting that these ice-binding agents are not antifreeze proteins that impart thermal hysteresis. For example, ice-binding proteins in the Antarctic nematode *Panagrolaimus davidi* do not exhibit thermal hysteresis, but can still modify ice growth by inhibited recrystallization (Wharton et al., 2005). Further studies may reveal a role for these ice-binding agents in extremely cold-tolerant populations of *P. rapae*.

5. Conclusions

We demonstrated that *P. rapae* can be freeze-avoidant, freeze-tolerant, or partially freeze-tolerant, and that cold tolerance strategy varies with developmental stage, population and acclimation. Notably laboratory acclimations failed to induce freeze tolerance in diapause pupae from Yakutsk (Russia), a population that is historically freeze-tolerant when overwintering in natural conditions. Cold tolerance was surprisingly invariable among populations when we acclimated pupae from Yakutsk (extreme winters) and London, Canada (mild winters) to common-garden conditions. However, exposure to subzero temperatures induced plastic responses (e.g. increased cryoprotectant concentrations) in Yakutsk, but not London, pupae. We suggest that this plasticity is important for increasing cold tolerance in populations of *P. rapae* that survive extreme winters (i.e. in Yakutsk).

Abbreviations

ANCOVA, analysis of covariance

ANOVA, analysis of variance

CTS, cold tolerance strategy

GC-FID, gas chromatography-flame ionization detection

GC-MS, gas chromatography-mass spectrometry

INA, Ice-nucleating agent

LC-MS, liquid chromatography-mass spectrometry

PC, principal component

PCA, principal component analaysis

SCP, supercooling point

$\dot{V}CO_2$, rate of carbon dioxide emission

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Declaration of interest

None. The authors have no competing interests to declare.

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Data availability

All data collected in this study are available in the supplementary data files.

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