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Metabolomes of bumble bees reared in common garden conditions suggest constitutive differences in energy and toxin metabolism across populations

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ABSTRACT

Cold tolerance of ectotherms can vary strikingly among species and populations. Variation in cold tolerance can reflect differences in genomes and transcriptomes that confer cellular-level protection from cold; additionally, shifts in protein function and abundance can be altered by other cellular constituents as cold-exposed insects often have shifts in their metabolomes. Even without a cold challenge, insects from different populations may vary in cellular composition that could alter cold tolerance, but investigations of constitutive differences in metabolomes across wild populations remain rare. To address this gap, we reared *Bombus vosnesenskii* queens collected from Oregon and California (USA) that differ in cold tolerance (CT_{min} = -6 °C and 0 °C, respectively) in common garden conditions, and measured offspring metabolomes using untargeted LC-MS/MS. Oregon bees had higher levels of metabolites associated with carbohydrate (sorbitol, lactitol, maltitol, and sorbitol-6-phosphate) and amino acid (hydroxyproline, ornithine, and histamine) metabolism. Exogenous metabolites, likely derived from the diet, also varied between Oregon and California bees, suggesting population-level differences in toxin metabolism. Overall, our results reveal constitutive differences in metabolomes for bumble bees reared in common garden conditions from queens collected in different locations despite no previous cold exposure.

1. Introduction

Cold tolerance is a fundamental trait linked to distributions of diverse animals (Andersen et al., 2015; Bishop et al., 2017; Sunday et al., 2019). Variation in cold tolerance among populations and species across space and among individuals through time, from acute responses to acclimation to seasonal variation, can inform our understanding of organismal ecology both now and under changing future climates (Campbell-Staton et al., 2017; Seebacher et al., 2015). Differences in cold tolerance depend on cellular-level mechanisms that are perhaps best understood for insects and provide a compelling opportunity to connect genotype to phenotype within the context of broader ecological implications (Brankatschk et al., 2018; Pimsler et al., 2020; Teets and Denlinger, 2013; Treanore et al., 2020).

Differences in physiological traits underlying cold tolerance variation across populations often have a genetic basis (e.g., Clark and Worland, 2008; Ma et al., 2020; Norry et al., 2007; Rako et al., 2007). Additionally, changes in gene expression have been linked to differences in cold tolerance both in conjunction with genetic differences (e.g., Ma et al., 2020; Parker et al., 2015) and in cases where there is little detectable genetic differentiation among populations (Pimsler et al., 2020; Sørensen et al., 2007). For example, cold-acclimated *D. melanogaster* exposed to 6 °C for 6 days had enhanced cold tolerance (further depressed CT_{min}) in conjunction with increased expression of genes previously associated with cold tolerance (i.e., *upheld, Tpn25D, Frost*, HSP22, *SMP-30,* etc.; Clowers et al., 2010; MacMillan et al., 2016). During recovery from -10 °C for 2 h, over a hundred genes related to cytoskeleton structure, organization, and cell shape were differentially expressed in *Sarcophaga bullata* (Teets et al., 2012), further coupling differential gene expression to cold tolerance and underlying ion

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homeostasis. Beyond gene expression alone, post-transcriptional changes expressed via proteomes may ultimately influence cold tolerance as elevated gene expression doesn't always result in changes in proteins (Tomanek, 2011). In lab-reared *D. melanogaster* selected for stress resistance, 118 proteins differed in abundance between cold shock resistant and control populations (Sørensen et al., 2017).

In concert with or independent of shifts in gene expression, changes in key metabolites that interact with membranes and proteins, thereby influencing cellular function, can also affect cold tolerance (Toxopeus and Sinclair, 2018). For example, sugars, i.e., trehalose (Koštál et al., 2011) and sucrose (Colinet et al., 2012; Kimura, 1982; Olsson et al., 2016), polyols (sorbitol; Colinet et al., 2012), and free amino acids, such as proline (Koštál et al., 2011; MacMillan et al., 2016) and alanine (Olsson et al., 2016), are elevated in *Drosophila* in response to cold acclimation. *Drosophila* species that differ in cold tolerance (LTe_{50}: the temperature that results in 50% mortality) had distinct metabolomes after exposure to 0 °C for 4 h (Olsson et al., 2016). Additionally, cold-hardy *D. melanogaster* have fewer changes in metabolite concentrations before and during exposure to cold compared to cold-susceptible populations, with hardier populations having lower concentrations of metabolites from the start, except for few that include phosphorylcholine and histidine (Williams et al., 2014).

Although many studies have explored associations between 'omics profiles and variation in cold tolerance, few have examined how constitutive differences among *natural populations* expressed *in the absence of cold exposure* may prime cold-adapted organisms to better tolerate cold (but see López et al., 2002; Martínez-Fernández et al., 2008). Perhaps the best evidence for constitutive priming comes from laboratory studies of *D. melanogaster* lines artificially selected for cold tolerance. Early work found that cold-selected lines had constitutively higher concentrations of glycogen, triacylglycerols and total protein (Chen and Walker, 1994) as well as of proline (Misener et al., 2001). Other fly lines selected for resistance to cold shock showed no constitutive differences in gene expression relative to controls (Sørensen et al., 2007); but follow-up proteomic analyses revealed over 100 differentially regulated proteins, including several heat shock proteins, suggesting that constitutive differences in molecular chaperone abundance may facilitate cold resistance (Sørensen et al., 2017). Furthermore, metabolomics revealed constitutively higher maltose and histidine and decreased free amino acids in cold-selected lines (Malmendal et al., 2013). And cold-hardy lines have been found to have larger, more connected metabolic networks than cold-susceptible lines (Williams et al., 2014). Together, these studies suggest that constitutive differences in 'omics may play an important role in population variation in cold tolerance. However, few studies have measured these differences for wild populations (see López et al., 2002; Martínez-Fernández et al., 2008) while also accounting for potential rearing (e.g., Kristensen et al., 2016) and acclimation (e.g., Colinet et al., 2013) effects, which can be pronounced.

Bumble bees (genus *Bombus*) are diverse and broadly distributed, with species and populations experiencing pronounced differences in climate. Recent work has found that cold tolerance (CT_{min}) differs strikingly across populations of the yellow-faced bumble bee (*Bombus vosnesenskii*; Pimsler et al., 2020), despite little population structure (Jackson et al., 2018), suggesting that cold tolerance is locally adapted. Genomics and transccriptomics studies suggest signatures of selection on cold tolerance across the range of the species associated with neural and muscular function, ion transport, signaling, and channel maintenance (Jackson et al., 2018; Pimsler et al., 2020). Common-garden reared bumble bees reared from queens collected from different populations showed strong gene expression differences in response to cold exposure: northern high-altitude populations upregulated expression of genes linked to membrane fluidity and ion channel function (e.g., *flotillin-1, Shal*; Pimsler et al., 2020). Similarly, genes related to carbohydrate metabolism were up-regulated in high-elevation species relative to their lower elevation counterparts (Liu et al., 2020). Yet, to what extent differences in cold tolerance across populations may also reflect differences (constitutive and induced) in cellular metabolites is unclear.

Because insect body compartments (head, thorax, and abdomen) differ in functions and associated tissue types, they may differ in cellular composition and metabolites associated with cold tolerance. For example, in the head the brain triggers glycerol production immediately after cold exposure (Yoder et al., 2006). In the thorax, the structure of muscle cellular membranes can vary with temperature, facilitating maintenance of membrane potential (preventing, for example, cold-induced cellular depolarization; Bayley et al., 2020). In the abdomen, ion homeostasis depends on the balance of secretion of ions into the hemolymph by the Malpighian tubules and reabsorption of ions by the hindgut; cold-tolerant species better maintain ion homeostasis in part due to the continued function of these key tissues (Andersen et al., 2017; Overgaard et al., 2021; Yerushalmi et al., 2018). Nevertheless, some tissues are found in multiple compartments. For example, neuromuscular junctions, the function of which strongly affects cold tolerance (reviewed in MacMillan and Sinclair, 2011; Overgaard and MacMillan, 2017), are found throughout the body. Similarly, the fat body, a key regulator of diverse processes as well as primary site of lipid storage, is predominantly found in the abdomen, but also extends to the thorax and head (Chapman, 1998), and hemolymph bathes all tissues (Heinrich, 2004). Similarities and differences in constitutive metabolites across body compartments may therefore suggest some of the processes underlying variation in cold tolerance.

We used a mass spectrometry-based untargeted metabolomics approach to answer the following question: do population differences in thermal tolerance traits reflect, in part, constitutive differences in cellular metabolites? Previous work revealed striking variation in cold tolerance across bumble bee populations (Fig. 1), with common-garden reared workers tolerating ~6 °C cooler temperatures when reared from queens collected in Oregon relative to those reared from queens collected in California (Pimsler et al., 2020). We captured wild spring *B. vosnesenskii* queens from these two populations, established colonies in common garden conditions to analyze the metabolomes of workers pulled directly from those colonies. This approach allowed us to detect constitutive differences in metabolomes between populations of bumble bees that differ in cold tolerance without exposure to cold during development or as adults.

2. Materials and methods

2.1. Bee collections and rearing

Wild queen bumblebees (*B. vosnesenskii*) were collected by net on April 21 and 22, 2017 at sites in Oregon (N 45° 69.6075′, W 121° 33.8828′, 70 m) and California (N 34° 22.0833′, W 117° 52.1111′, 2023 m), respectively (Fig. 1). Queens were kept on ice for transport to the USDA-ARS Pollinating Insects Research Unit (Logan, UT) where they were stimulated to initiate colonies as described in Oyen et al. (2021). Briefly, queens were placed in small chambers with fresh pollen and nectar substitute until they began to manipulate wax and lay eggs. After colonies reached approximately 40 individuals, they were transported to the University of Wyoming (Laramie, WY) where they were maintained in the same conditions and fed the same diet: 27 °C, 12:12 L:D cycle with ad libitum access to nectar and fresh pollen provided every 2–3 days.

2.2. Sample processing and analyte extraction

Five healthy workers with little to no wing wear (indicating they were of young age; Mueller and Wolf-Mueller, 1993) were taken directly from one colony per population and immediately flash frozen in liquid nitrogen. They were then sectioned into head, thorax, and abdomen on dry ice with sterile razor blades and fresh gloves used for each individual before being stored at -80 °C. Every step of the metabolite extraction method was performed with ice cold solvents and all processes were

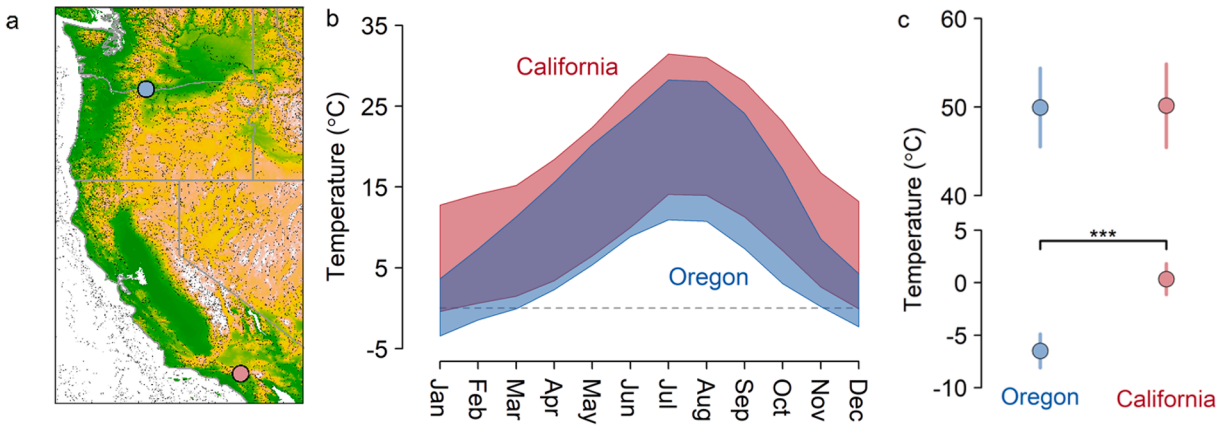

Fig. 1. Bumble bee cold tolerance differs between populations. a) Queen *Bombus vosnesenskii* were collected from two different sites in Oregon (blue) and California (red) b) that have distinct temperature profiles; c) offspring reared in common-garden conditions from collected queens differed in CT_{min} ($P < 0.001$, $F_{1,166} = 828.45$) but not CT_{max} ($P = 0.77$, $F_{1,182} = 0.0861$; Pimsler et al., 2020).

done on ice or in a 4 °C environment to minimize artifact formation or metabolite decomposition during extraction. Each bee segment was weighed and placed in 1.5 mL polypropylene microcentrifuge tubes. Methyl *tert*-butyl ether (MTBE) extraction solution, composed of 6:3:1 MTBE:methanol:water by volume, was added to the tube, at a solvent to sample ratio of 20 μL/mg of bee tissue. Each tissue was milled using the end of a sonicating wand for 1 min to break up the tissues, then sonicated with three sonication steps of 20 s each at 4 W root mean square (RMS) power, with a 20 s break between steps to prevent overheating, and continuously kept on ice. After all samples were sonicated, the capped tubes were vortexed for 30 min at 4 °C. Water was then added to each vial to induce phase separation, with water volume equal to 20% of the original MTBE solution volume. This was followed by centrifugation at 12,000 RPM for 20 min at 4 °C. Both the lower aqueous layers and upper organic layers of these samples were transferred to separate autosampler vials fitted with 250 μL volume reducing inserts for analysis of the individual samples. Pooled quality control (QC) extracts of the organic and aqueous fractions were prepared by combination of 50 μL aliquots of every sample into single vials, and vortexed 5 min at 4 °C for thorough mixing. Four individual pooled QC samples for each body segment of each phase were prepared for analysis by aliquoting 40 μL into autosampler vials fitted with 250 μL volume reducing inserts. The samples were chilled to -20 °C overnight to aid in additional protein precipitation prior to analysis. Two method blanks were prepared in parallel to the samples, differing only in that no tissue was added to the 1.5 mL micro centrifuge tubes at the beginning. All vials were centrifuged a final time (12,000 RPM centrifugation for 20 min at 4 °C) before LC-MS analysis.

2.3. Liquid chromatography

The stacked injection of biphasic extraction (SIBE) scheme was used (Broeckling and Prenni, 2018) and consisted of consecutive injections onto the reverse phase column under isocratic low eluent strength conditions (100% A) of first the organic fraction (1 μL) and then the aqueous fraction (1 μL), followed by the LC gradient solvent program. Solvent A was 99.9% water with 0.1% formic acid, and solvent B was 99.9% acetonitrile with 0.1% formic acid, with the following LC gradient elution program: 0% solvent B, increased to 1% B by 2 min, then a linear ramp up to 99% B in 33 min, held for 15 min to flush, then returned to 0% B by 51 min for re-equilibration. The volumetric flow rate was 50 μL/min on a C18 Reversed Phase column (Waters Aquity HSS T3 column; 1mmx1.7umx15cm) held at a constant temperature of 40 °C. Each sample, blank, and quality control was analyzed twice: first in positive ionization mode then immediately after in negative ionization mode to minimize evaporative losses of the sample solvent through the pierced septum. Follow up analyses were later performed on the aqueous layer of the Pooled QC samples by Hydophilic Interaction Liquid Chromatography (HILIC) to further investigate carbohydrate biomarkers of interest not fully resolved in the RPLC separation (Supplemental Fig. 1). These analyses were performed on a HILIC column (1 mm × 1.7 μm × 15 cm BEH Amide, Waters Corp.). Solvent A was 96% acetonitrile with 4% water, and solvent B was 100% water, each with 5 mM ammonium formate and 0.1% formic acid. The column was maintained at 40 °C and solvent flow was 40 uL/min with a 1 μL injection volume. The solvent gradient was: 10% B to start, linear ramp reaching 13% B at 34 min, linear ramp reaching 60% B at 55 min, held 60% for 6.5 min to flush, and re-equilibrated at 10% B for 13 min.

2.4. Electrospray Ionization (ESI) Mass Spectrometry

A hybrid quadrupole-Orbitrap mass spectrometer (Q-Exactive HF-X; ThermoScientific) fitted with a Heated-Electrospray Ionization (HESI; ThermoScientific) ion source with a 32 gauge spray capillary was used for all analyses. Each sample was analyzed sequentially in positive ion and negative ion modes. For positive ion mode, the HESI source spray capillary was held to +3.4 kV, MS inlet transfer capillary temperature of 250 °C, HESI probe temperature of 150 °C, sheath gas of 26, auxiliary gas of 9 (all gas flows arbitrary units), and the ion funnel RF level was set to 40. For negative ion mode, the HESI source spray capillary was held to -2.5 kV, MS inlet transfer capillary temperature of 250 °C, HESI probe temperature of 150 °C, sheath gas of 30, auxiliary gas of 10, and the ion funnel RF level was 40. Data was collected in the data dependent analysis mode (DDA; Top 5), generating both full scan (MS) and tandem MS (MS^2) spectral data, with a scanning range of m/z 80–1200. Full scans were collected at a resolving power setting of 120,000 (256 ms transients), with automatic gain control (AGC) set to 10^6 and maximum injection time of 100 ms. Tandem MS scans were collected at a resolving power setting of 15,000 (32 ms transients), AGC set to 10^5 and maximum injection time of 60 ms. The quadrupole was set with an isolation width of 1.2 Da in MS^2 and the High-energy Collisional Dissociation (HCD) cell was operated with stepped normalized collision energy (NCE) of 20, 30, and 40. A list of the top 200 most abundant ions present in method blank samples was placed on a permanent exclusion list, precluding them from being selected for MS^2 during sample analysis. Dynamic exclusion was turned on, with a time of 8 s. All ions with charge states greater than +2 were excluded from MS^2 measurements, along with isotopic ions (exclude isotopes: on).

2.5. Quality control

The pooled QC samples were analyzed before, during, and after the individual bee extract samples (every 5 injected samples) to monitor for retention time and signal drift over the time course of the instrumental analysis sequence and provide instrumental variance information for each detected metabolite. The injection order of all samples was randomized to eliminate bias due to analysis-order. Triplicate samples of each body region as well as for all QC samples meant that running all samples within the same instrument run required nearly two weeks of continues instrument time. Because these are relative comparisons, running samples across multiple instrument runs is problematic, necessitating tradeoffs in sample selection. We therefore opted to run three separate body regions for five individuals from each of two colonies reared from the two source populations, rather than to sample more individuals from more colonies which would have precluded differentiation among body segments.

2.6. Data processing and analyses

The goal of this work is to discover the metabolome differences, if any, between these two phenotypes. Consequently, all of the metabolite assignments were performed using a mass spectral database match only, which corresponds to a Metabolomics Standards Initiative (MSI) Level 2 annotation (Schymanski et al., 2014), and as a result, all compound annotations in this study are probable or tentative structures. For a selected number of metabolites, we provide further confirmation through the analysis of commercially available chemical standards (*vide infra*). All LC-MS/MS raw data files generated were converted to an Analysis base file (Abf) format (https://www.reifycs.com/AbfConverter/) and pre-processed in MS-DIAL (http://prime.psc.riken.jp/compms/msdial/main.html), where chromatographic features were detected, chromatogram retention times were aligned, gap filled, and blank filtered. Feature annotation was performed using a merged database file comprised of NIST17 and the databases publicly available through MS-DIAL (version 15 of the compiled databases, downloaded 12/16/2020). MS-DIAL results were exported as csv files for subsequent data visualization and multivariate analysis. For the metabolites maltitol, trimethyllysine, and sorbitol (Supplemental Fig. 1), reference standards were analyzed on the same instrumental platform to confirm identifications at the MSI Level 1 standard (Schymanski et al., 2014).

We assessed normality of critical thermal limits data with a Shapiro-Wilk's test. Though data were non-normal, sample sizes were large, $n > 50$, so we analyzed CT_{min} and CT_{max} across populations using ANOVAs (Fig. 1c; Pimsler et al., 2020). Redundant chromatographic features that had exact name matches after accounting for capitalization were

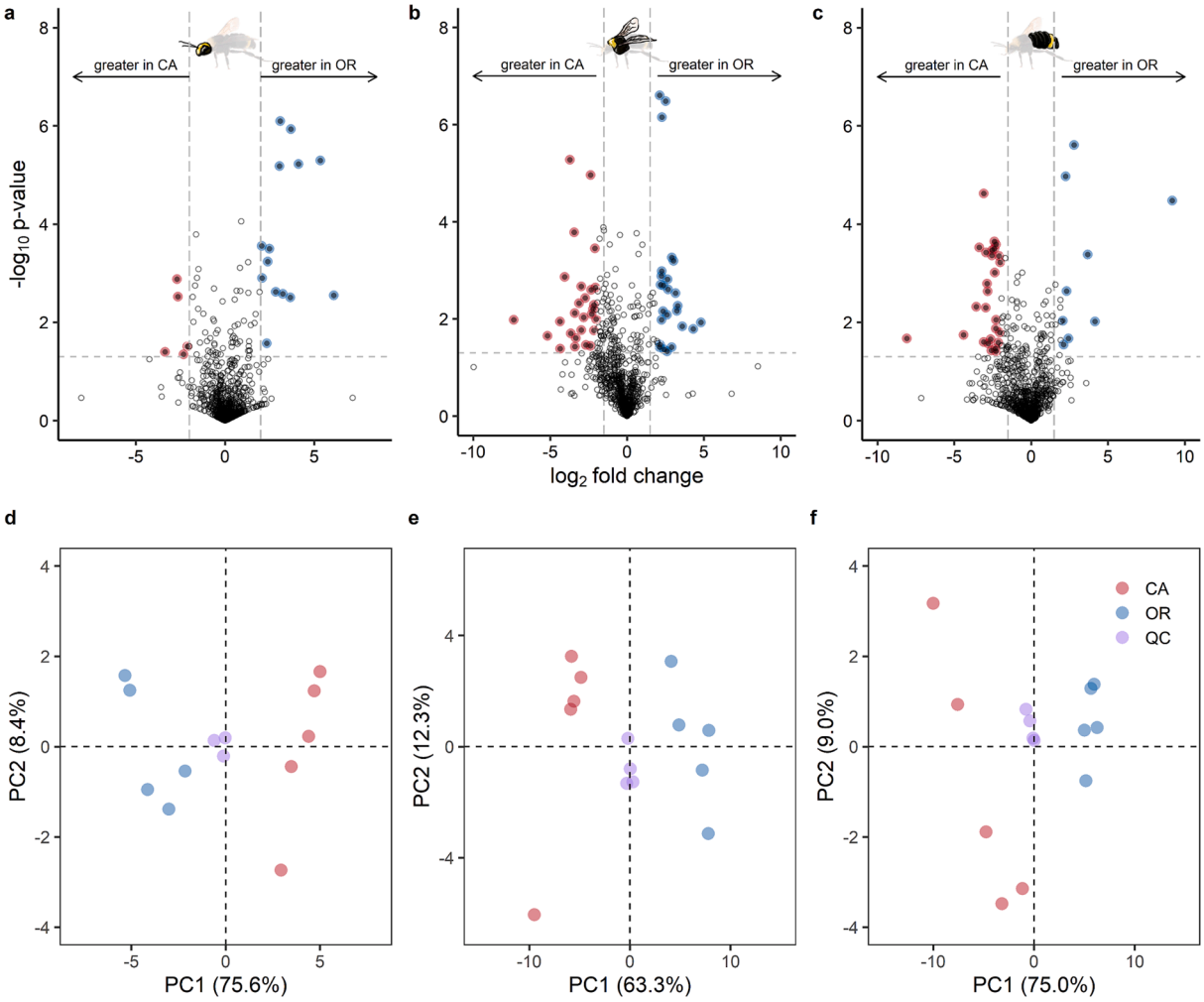

Fig. 2. Bumble bees reared in common garden conditions cluster by maternal population source based on differentiated metabolites. Volcano plots of all metabolites identified in the a) head, b) thorax, and c) abdomen of bumble bee workers reared in common-garden conditions from queens collected in OR and CA. Features most strongly differentiated between populations (adjusted p-values < 0.05 and $\log_2$ fold change > 2) are colored blue (upregulated in OR) and red (upregulated in CA). Based on these features, principal component analyses revealed distinct clusters of OR (blue) and CA (red) bees for d) head, e) thoracic, and d) abdominal segments with QC samples (purple) around zero and in between the two groups for all segments.

grouped into single features. Features that had greater than 25% coefficient of variation across pooled QC samples or that were absent from pooled QC samples were excluded. Features found in at least one sample from both populations were included in the volcano plots for each body segment (Fig. 2a,b,c), while those that met QC requirements with a *t*-test derived p-value < 0.05 but were found in only one of the populations were analyzed separately (Supplemental Table 1). Features of interest meeting significance and fold change thresholds ($P < 0.05$ and $\log_2$ fold change > 2) were analyzed in segment-specific PCA plots to compare QC and individual differentiation across populations using *fviz_pca_ind()* from the *factoextra* package in R (Kassambara and Mundt, 2020). QC samples were tightly clustered and intermediate between the two populations (Fig. 2d,e,f), indicating minimal sampling error or instrument drift. In the results, we compared significantly different features detected in every individual with fold changes greater than two between populations and condensed feature names indicated by a star with full names retained in Supplemental Table 2 (Fig. 3). To identify metabolites of interest that have weaker signals, we also compared features with reduced fold change (1.5 instead of 2; Supplemental Figs. 4 and 5) and/or detected in fewer individuals (3 + or all individuals; Supplemental

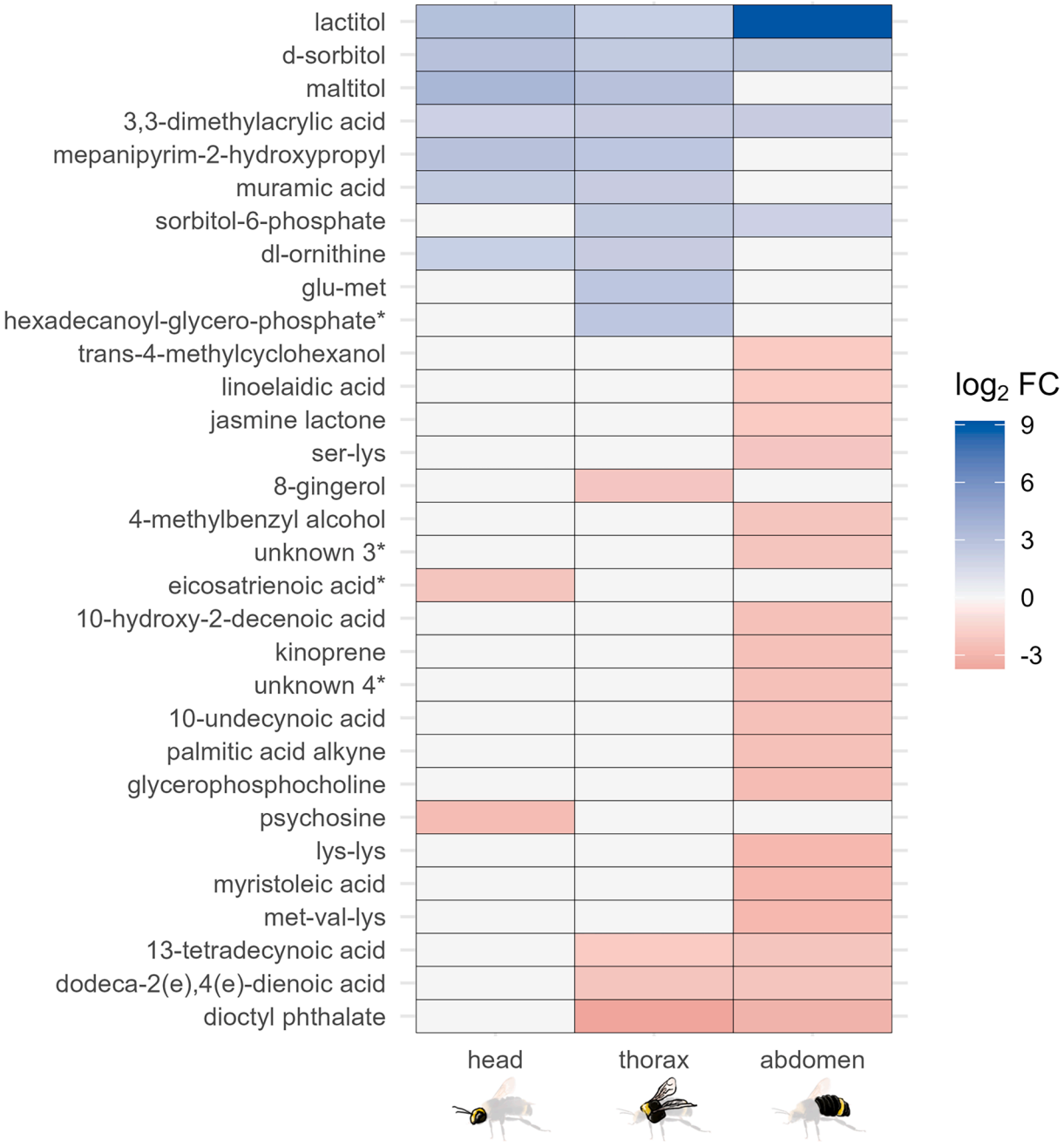

Fig. 3. Key features differentiated between OR and CA bumble bees across body segments. Log_2 fold change of each metabolite detected in every individual differed across the head (left), thorax (center), and abdomen (right) between OR and CA bumble bees; increasing or decreasing fold change was metabolite-specific with those of increasingly blue saturation found in greater magnitudes in the OR bees and increasingly red saturation indicating greater magnitudes found in the CA bees. White boxes represent metabolites that were either unidentified in that body segment in one or both populations or fell short of QC, significance, or fold change thresholds (see text for details). Shortened metabolite names are indicated by "*" with full names provided in Supplemental Table 2.

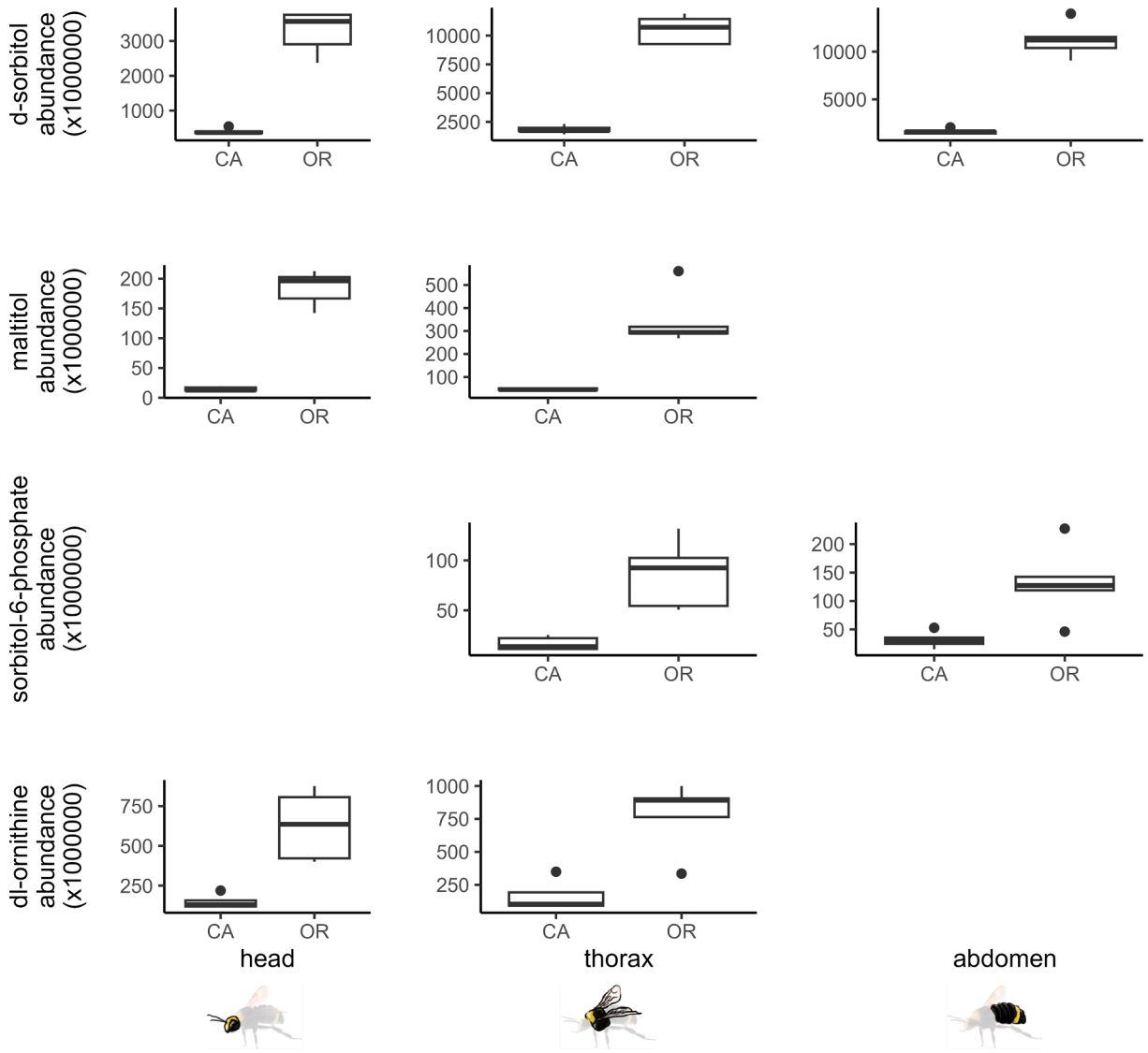

Fig. 4. Bee populations maintained constitutive differences in metabolites related to carbohydrate and amino acid metabolism. Metabolites associated with carbohydrate and amino acid metabolism identified in the head (left), thorax (center), and abdomen (right) were more abundant in the OR population (all adjusted $P < 0.01$).

Figs. 4 and 5, respectively), which we refer to in the text as "less stringent" (Supplemental Table 3). We used pairwise t-tests with a Bonferroni correction to assess the relationship between the abundance of each metabolite of interest across source population and body segment for metabolites of interest (Fig. 4; R Core Team, 2021).

3. Results

3.1. Summary of features

We detected a total of 46,606 features across all bumble bee samples, with 1,341 of those varying less than 25% in the pooled QCs and therefore retained for subsequent analyses. A subset of these features were detected in at least one worker from both populations and at least one QC sample: 987 features in the head, 832 in the thorax, and 948 in the abdomen (Fig. 2a,b,c). Of these, 19, 52, and 36 features in the head, thorax, and abdomen, respectively, had significant and more than two-fold differences in abundance between the two source populations (Fig. 2d,e,f). Features absent from one population but found in the other totaled 76, 111, and 77 from the head, thorax, and abdomen respectively; of those, 37 features differed significantly across populations ($P < 0.05$ and $\log_2$ fold change > 2) and were found in at least one QC sample: 8 in the head, 18 in the thorax, and 12 in the abdomen (Supplemental Table 1).

3.2. Carbohydrate metabolism

Overall, metabolites related to carbohydrate metabolism were more abundant in the more cold-tolerant OR bees. D-sorbitol and lactitol were more abundant across all segments of the OR population (Fig. 3, Fig. 4, Fig. 5). Sorbitol-6-phosphate was more abundant in the head and thorax of OR bees (Fig. 3; Fig. 4), but was minimally detected in CA head samples (Supplemental Table 2), indicating a robust pathway leading to glycolysis via carbohydrate metabolism by the OR population (Fig. 5). Additional carbohydrate-based metabolites differed between populations when we reduced stringency (Supplemental Table 3). For example, maltitol was more abundant in the head and thorax of OR bees (Fig. 3, Fig. 4) and in the abdomen (Supplemental Fig. 2; Supplemental Fig. 5; Supplemental Table 2) possibly via association with sorbitol (Fig. 5; Supplemental Fig. 1). Additionally, palatinose was more abundant in the head and thorax of OR bees, but was only detected in two and one of those respective segments in the CA bees (Supplemental Table 2).

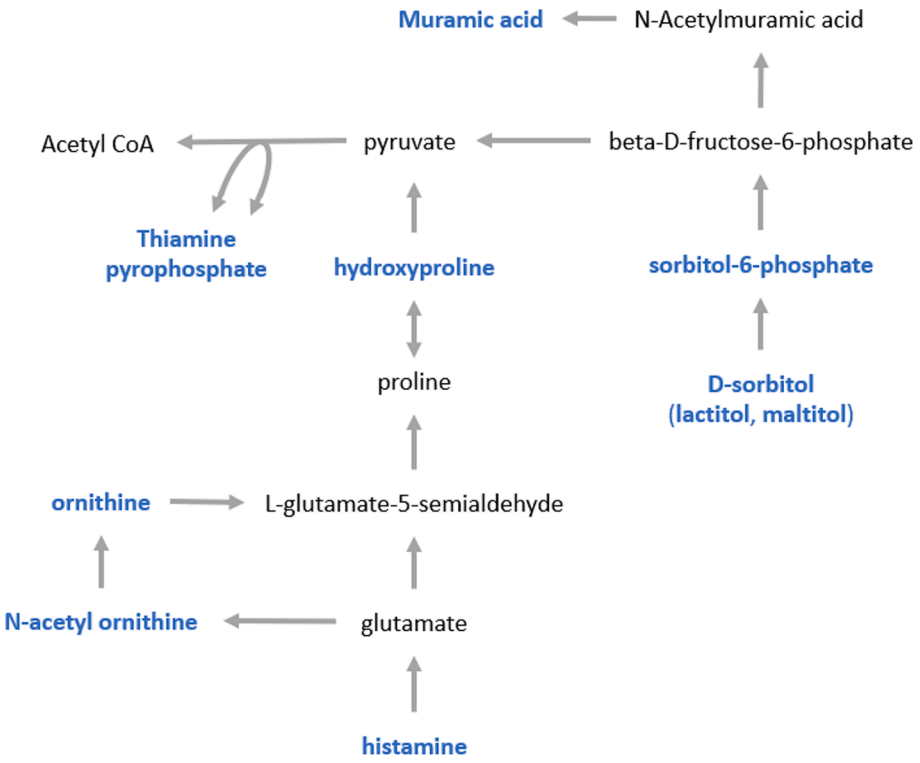

Fig. 5. OR bees had greater abundances of metabolites related to carbohydrate and amino acid metabolism. Constitutive differences in carbohydrate metabolism between common-garden reared bees from cold-adapted and warm-adapted populations. OR bees had higher levels of sorbitol and sorbitol-6-phosphate which can ultimately feed into glycolysis. OR bees had higher concentrations of histamine, N-acetyl ornithine, ornithine, hydroxyproline, and thiamine phosphate which can ultimately feed into the citric acid cycle. Color indicates higher abundance in OR (blue).

Palatinose, a disaccharide, may serve as a precursor to glucose which can be converted into sorbitol, ultimately leading to glycolysis (Fig. 5). Additionally, thiamine pyrophosphate, a cofactor including for carbohydrate metabolism, was more abundant in abdomen of OR bees (Fig. 5; Supplemental Table 2). The most stringent restrictions (Supplemental Table 3) revealed that samples grouped by maternal location of origin were physiologically distinct (Supplemental Fig. 3). Interestingly, the metabolites most upregulated in the head of OR bumble bees were notably more upregulated than those in the CA bees (lower p-values and higher fold changes; Fig. 2a).

3.3. Amino acid-related compounds

Several amino acid-related metabolites associated with protein metabolism were more abundant in the OR bees. Ornithine and muramic acid (an amino acid sugar) were more abundant in head and thorax of OR bees (Fig. 3; Fig. 5). Additionally, Glu-Met was more abundant in the thorax of OR bees (Fig. 3) but was absent from the abdomen of CA bees (Supplemental Table 1). CA bees had more poly-amino acid metabolites (short peptides), including Met-Val-Lys, Lys-Lys, and Ser-Lys, in the abdomen than those from OR (Fig. 3). After reducing stringency, Arg-His, Glu-Ala-Arg, and Glu-Thr were also more abundant in the abdomen, thorax, and head and thorax, respectively, of CA bees (Supplemental Fig. 2; Supplemental Fig. 4; Supplemental Table 3), while n-. alpha.-acetyl-l-ornithine and *trans*-4-hydroxy-l-proline were more abundant in the thorax and the head and thorax, respectively, of OR bees (Supplemental Fig. 5; Fig. 5). Lastly, histamine was more abundant in the thorax (Supplemental Table 2) and abdomen of OR bees (Supplemental Fig. 4; Supplemental Fig. 5; Fig. 5). While the direct influence of these amino acids and related compounds on cold tolerance remains unclear and their abundance may also be associated with other pathways not discussed here, together they may reveal enhanced protein metabolism in the OR population (Fig. 5).

3.4. Lipid-related compounds

Under the most stringent filters, CA bees generally had greater abundances of lipid metabolites across all body segments (Fig. 3), though they occurred mostly in the abdomen, followed by the thorax, and with the fewest differences detected in the head. The diverse lipid-related features were not clearly linked to a single pathway, so here we summarize differences in individual features. Medium to long chain unsaturated fatty acids were more abundant in the abdomen (dodeca-2 (e),4(e)-dienoic acid, 13-tetradecynoic acid, myristoleic acid, 10-undecynoic acid, 10-hydroxy-2-decenoic acid, and linoelaidic acid), thoraces (dodeca-2(e),4(e)-dienoic acid and 13-tetradecynoic acid), and head (eicosatrienoic acid) of the CA population, with one saturated fatty acid more abundant in the CA abdomen as well (palmitic acid alkyne; Fig. 3). One glycerophospholipid, 1-hexadecanoyl-2-*sn*-glycero-3-phosphate, was more abundant in the thorax of OR bees (Fig. 3). Glycerophosphocholine was less abundant in the abdomen of OR bees (Fig. 3; Fig. 4) and also in the head under less stringent fold change thresholds (Supplemental Fig. 4). After reducing stringency, 10-hydroxy-2-decenoic acid was more abundant in the thorax of CA bees (Supplemental Fig. 2). Additionally, 2-hydroxypalmitic acid, a long chain fatty acid, was more abundant in the abdomen of CA bees (Supplemental Fig. 2) with palmitic acid alkyne also appearing in greater abundance in the thorax and abdomen of CA samples (Supplemental Fig. 4).

3.5. Exogenous compounds

Several diet-associated features were more abundant in the abdomen and thorax of CA bees. Some aromatic metabolites were detected in every sample, with CA bumble bees containing more 8-gingerol and jasmine lactone in the thorax and abdomen, respectively (Fig. 3). Additional diet-related compounds found in all individuals from one population and absent from the other included rutin and 2,5-

dihydroxyacetophenone, which were greater in CA thoraces and abdomens, respectively, and piperine, which was greater in the abdomen of OR bees (Supplemental Table 1).

Exogenous compounds likely acquired through the diet, including plasticizers, pesticides, and herbicides, varied across populations and segments (Fig. 3; Supplemental Fig. 6). Dioctyl phthalate and mono-2-ethylhexyl phthalate (MEHP) were greater in the thorax and abdomen of CA bees with MEHP absent from OR bees (Fig. 3; Supplemental Fig. 6; Supplemental Table 1). Kinoprene was detected in every abdomen across populations, with the CA bees containing significantly more (Fig. 3; Supplemental Fig. 6). The CA thoracic samples also contained more fentrazamide, though it was only detected in four of the five OR thoraces (Supplemental Fig. 2; Supplemental Table 2). Conversely, isoxadifen-ethyl was detected in every OR head but was absent from CA bees (Supplemental Fig. 6; Supplemental Table 1) and mepanipyrim-2-hydroxypropyl was found in every head and thorax across populations but was more abundant in the OR bees (Fig. 3; Supplemental Fig. 6; Supplemental Table 2). It is worth noting that, with the exception of a few carbohydrates (Supplemental Fig. 1), these signals were not confirmed with pure standard compounds so their identification is based on current database matches (corresponding to a MSI Level 2 annotation–see *Data Processing and Analyses* in the methods).

4. Discussion

More cold tolerant bees reared from high elevation OR queens had constitutively elevated levels of a number of metabolites associated with carbohydrate metabolism (Fig. 5). Similarly, transcripts related to glycolysis and oxidative phosphorylation were upregulated in high elevation bumble bees compared to low elevation species (Liu et al., 2020). Temperature-associated shifts in carbohydrate metabolism have also been seen in other insects. For example, proteomes of adult *D. melanogaster* reared in hot conditions (31 °C) revealed down-regulation of carbohydrate metabolism and energy production compared to adults reared at room temperature (25 °C; Kristensen et al., 2016). *D. simulans* reared in hot conditions had reduced expression of genes related to glycolysis and oxidative phosphorylation (Mallard et al., 2020). Transcriptomes of ticks (*Chymomyza costata*) recovering from cold shock suggested upregulation of carbohydrate metabolism relative to control animals (Rosendale et al., 2022). And cold-selected lines of *D. melanogaster* had higher glycogen concentrations than control lines (Chen and Walker, 1994), again suggesting increased carbohydrate metabolism in response to cold. Although we did not detect differences in glycogen or trehalose, other carbohydrate related metabolites, including several sugar alcohols like sorbitol, maltitol, and lactitol (Fig. 4; Fig. 5), could ultimately increase energy production via glycolysis.

Sorbitol can also function as a cryoprotectant that can mitigate chill injury when organisms face low temperatures across short or long timescales (Denlinger and Lee Jr., 2010; Teets and Denlinger, 2013). For example, sorbitol concentration rapidly increases in flesh flies (*Sarcophaga crassipalpis*) to induce cold hardening within hours (Michaud and Denlinger, 2007) and increases seasonally in European firebugs (*Pyrrhocoris apterus*) to prepare for overwintering (Tomcala et al., 2006). Sorbitol also increases in cold-acclimated fruit flies (*D. melanogaster*) when exposed to chronic cold and in goldenrod gall fly hemolymph (*Eurosta solidaginis*) compared to their warm-acclimated counterparts (Colinet et al., 2012; Storey et al., 1986). The role of maltitol in insect physiology is not well understood, but maltose networks, possibly associated with maltitol, are more connected in cold-hardy *D. melanogaster* compared to cold-susceptible flies (Williams et al., 2014). Curiously, lactitol, a sugar alcohol, was found in greater abundance in every segment and up to nine times higher in OR bees (Supplemental Table 2). Lactitol is also not commonly associated with insect cold tolerance, though has been detected in larval *D. melanogaster* (Williams et al., 1991). Outside of the scope of insects, lactitol was identified as one of several metabolites differentiating the metabolomes of cold-exposed tea plants (*Camellia sinensis L.*) to counterparts that were cultivated in warmer mean temperatures (Shen et al., 2015). The high abundance of lactitol observed across segments, though especially in the abdomen, of the OR bumble bees warrants further investigation prior to conclusively attributing the polyol to feeding and digestion, metabolism, or cold tolerance.

The upregulation of sugar alcohols across all body segments of the OR bees could indicate that some bumble bees may be predisposed to cold hardening despite the lack of cold exposure in their lifetimes. Follow-up analyses using hydrophilic interaction liquid chromatography (HILIC) suggest carbohydrate biomarkers may not be fully resolved by the RPLC separation (Supplemental Fig. 1). Nonetheless, population-level differences in sugar and polyols have been recorded in other insects during cold exposure. For example, both temperate and tropical locust eggs had increased glucose and sorbitol levels after cold exposure, and the more cold-tolerant temperate population had increased mannitol, glucose, and sorbitol when exposed to extreme cold (Wang et al., 2010). Our findings of increased sugar alcohol abundance in cold-tolerant OR bees agree with these previous studies and suggest that underlying levels of polyols differ across populations even in the absence of cold exposure. Yet during cold exposure, transcriptomic analyses suggest that cold-tolerant OR *B. vosnesenskii* have lower abundance of an alpha-glucosidase-like transcript, which likely functions to cleave disaccharides (Pimsler et al., 2020). We would expect the OR population to have more alpha-glucosidase-like transcripts since our results suggest that all other metabolites associated in the corresponding carbohydrate pathway are elevated in OR bees. It's possible that a cold challenge may induce CA bees to upregulate the transcripts to compensate for already depressed carbohydrate metabolism, though that idea would need further study.

Trehalose is a common disaccharide found in bumble bees (Chapman, 1998) and many other insects (Beenakkers et al., 1984; van der Horst et al., 1978; Wyatt, 1967) as a primary energy source for function, like flight, and as a stress response (reviewed in Crowe et al., 2001). Yet, trehalose did not differ across populations in our study. We expected more trehalose in the cold-tolerant OR bees since trehalose has been associated with enhanced cold tolerance: increased concentration of low molecular weight solutes, like trehalose, further depresses supercooling points (the temperature at which internal ice nucleation occurs; Salt, 1961; Sinclair et al., 2015) and melting points given their colligative properties (Denlinger and Lee Jr., 2010; Holmstrup et al., 2002; Zachariassen, 1985). Trehalose increases in freeze-avoidant Arctic springtails (*Onychiurus arcticus*) during exposure to cold temperatures with concurrent decreases in water content resulting in enhanced cold tolerance (Worland et al., 1998). While trehalose decreases in chronically cold-acclimated *D. melanogaster* adults compared to warm-acclimated flies (MacMillan et al., 2016), cold-acclimated larvae have elevated trehalose (Koštál et al., 2011) as do *Drosophila* species exposed to acute cold (4 h at 0 °C; Olsson et al., 2016). Increased trehalose abundance in insect hemolymph may be derived from the trehalose-specific transporter (TRET1) which is upregulated in the larval fat body of midges (*Polypedilum vanderplanki*) when exposed to environmental stress (i.e., increased desiccation and salinity exposure; Kikawada et al., 2007). Importantly, use of RPLC in this study made differentiating between structurally similar molecules like turanose and trehalose difficult because they were not well retained and separated, and thus we relied on the MS/MS spectral database search algorithm to indicate which signals correspond with which isomers. However, review of the MS/MS spectral database suggests that MS/MS may poorly differentiate some sugars because of their nearly identical spectra. Follow-up analyses on thoraces using HILIC separation, albeit with aged samples stored at only -20 °C, indicated that seven disaccharides with highly similar MS/MS spectra were detected, with five of them clearly upregulated in the OR bumble bees (Supplemental Fig. 1). Future work that focuses on these difficult to separate carbohydrates may reveal

biologically important differences across populations that we failed to detect.

While amino acids were variable across populations and segments, several metabolites associated with amino acid metabolism, like ornithine, *trans*-4-hydroxy-l-proline, and histamine, which can be intermediates prior to the citric acid cycle, were more abundant in OR bees (Fig. 3; Supplemental Fig. 4; Fig. 5). Beyond this potential effect on metabolism (Fig. 5), the potential influence of these amino acids on population differences in bumble bee cold tolerance remains unclear. Amino acids can serve as cryoprotectants (Denlinger and Lee Jr., 2010). Many metabolomic studies have found that proline (Koštál et al., 2011; Moos et al., 2022; Teets et al., 2012), alanine (Michaud and Denlinger, 2007; Olsson et al., 2016), and glutamate (Koštál et al., 2011; Moos et al., 2022) are linked to insect cold tolerance. Although detected, proline and alanine were not significantly different between OR and CA bees. However, *trans*-4-hydroxy-l-proline, a proline intermediate (Fig. 5), was more abundant in the head and thorax of OR bees when stringency was lowered (Supplemental Fig. 4), Pro-Leu was detected in the abdomen of three CA bees but was undetected in OR bees (Supplemental Table 1), and Glu-Ala-Arg was more abundant in CA thoraces (Supplemental Table 1). Glutamate was not detected in the feature list that met QC standard deviation thresholds, but glutamine and glutamic acid, precursors of glutamate and glutamine, respectively, (Schousboe et al., 2014) were identified; they too, however, were not significantly different across populations. Glu-Met, a dipeptide composed of L-glutamic acid and L-methionine joined by a peptide linkage, was more abundant in the thorax of cold-tolerant OR bumble bees (Fig. 4). Glutamic acid, a precursor to proline (Vogel and Davis, 1952) and glutamine (Krebs, 1935), increases in cold-acclimated wheat weevils (*Sitophilus granaries*) and bark beetles (*Cryptolestes ferrugineus*; Fields et al., 1998), while glutamine increases in cold-tolerant *Drosophila* species after acute (4 hrs at 0 C; Olsson et al., 2016) and chronic cold exposure (6 days at 6C; MacMillan et al., 2016). Ornithine, another precursor to proline (Delauney and Verma, 1993), was more abundant in the OR bumble bee head, thorax, and abdomen (Fig. 4). Interestingly, ornithine decreases in flesh flies after acute cold exposure (8 h at 4 °C in *S. crassipalpis*; Michaud and Denlinger, 2007). Perhaps, the elevated constitutive levels of ornithine in the OR bees would provide for rapid proline synthesis in response to a cold challenge, though this idea is merely speculative and would need to be tested. Nonetheless, the differential accumulation of amino acid-based precursors to cryoprotectants and di- and tripeptides containing cryoprotectants in the absence of any cold exposure suggests the baseline metabolome of OR bees may enhance amino acid metabolism and energy production via the citric acid cycle (Fig. 5). Conversely, their presence may simply reflect metabolic intermediates of diet processing related to feeding, digestion, absorption, or metabolism that differs across populations.

Glycerophosphocholine can serve as an osmolyte, produced from mammalian renal cells to regulate cellular osmolality when salinity increases (Zablocki et al., 1991), and as a metabolite that maintains lipid membrane structure across variable conditions. During cold challenges, glycerophosphocholine is often replaced with glycerophosphoethanolamine, a lipid metabolite with a smaller headgroup that increases disorganization to maintain cellular membrane fluidity necessary for function during low temperatures (Denlinger and Lee Jr., 2010; Hazel, 1995; Overgaard et al., 2008; Tomcala et al., 2006). Interestingly, OR bees had a lower abundance of glycerophosphocholine (Fig. 3); its diminished abundance may suggest that less glycerophosphocholine is more conducive to increased ratios of glycerophosphoethanolamine to glycerophosphocholine when challenged by cold as exchanging fewer lipids may be energy saving. Characterizing the change in glycerophosphocholine abundance from room temperature to cold conditions for animals from the two populations may reveal population-level differences in cellular level responses to changing temperatures.

Because OR and CA bees were provided with the same diet and feeding regime, we expected populations to contain similar profiles of environmentally-derived (exogenous) metabolites; however, relative abundances in aromatic and flavor-based metabolites differed between body compartments and populations (Fig. 3). These curious findings may suggest that feeding behavior, digestion, absorption, and/or metabolism of substances varied for bees from the two populations despite being fed the same diet and kept in the same conditions.

Other exogenous metabolites also differed in abundance across body segments and populations: disoctyl phthalate, a natural odor found in plants as well as a plasticizer and environmental hazard in liquid form (PubChem, n.d.), and mono-2-ethylhexyl phthalate (MEHP), a metabolite of di-2-ethylhexyl phthalate, another toxic plasticizer (Erkekoglu et al., 2010), were both more abundant in the thorax and abdomen of CA bees (Fig. 3) with MEHP not detected in OR bees (Supplemental Table 1). The plasticizers could have come from chewing on the plastic nest boxes. Alternatively, plasticizers could have leached into nectar from plastic storage containers or the pollen collected by honey bees could have been tainted with pesticides and plasticizers. Outside of the lab, the presence of microplastics in pollinators like honey bees (Edo et al., 2021) and solitary bees (Allasino et al., 2019; MacIvor and Moore, 2013) has recently received increased interest (de Souza Machado et al., 2018) as they can have cascading effects on microbiota, immunity, and gene expression when consumed (Al Naggar et al., 2021). Additionally, kinoprene, an insecticide that inhibits insect development (Hamaidia and Soltani, 2014) and is lethal at high doses (Mommaerts et al., 2006) was identified in the thorax and abdomen of both populations, with the CA bees containing more than the OR population (Fig. 3). While CA bees overall had more harmful substances across segments, mepanipyrim-2-hydroxypropyl, a pesticide found in agricultural systems (Fernandes et al., 2012), was more abundant in both head and thoracic segments of OR bees (Fig. 3) and isoxadifen-ethyl, a herbicide safener, was only detected in OR heads (Supplemental Table 1). The differential abundance of disoctyl phthalate, MEHP, kinoprene, mepanipyrim, and isoxadifen-ethyl, stemming either from nectar, pollen, or the chamber itself, may suggest differences in feeding behavior, or alternatively, could indicate differences in metabolism across populations. Further investigation is required to identify population-level differences in dietary selection, digestion, absorption, and metabolism of pesticides and plasticizers across populations in laboratory and wild settings. With few studies in wild settings, future research characterizing physiological responses of bumble bees in the presence of such environmentally-derived metabolites may reveal the effects of these perhaps ubiquitous toxicants on native pollinators.

In conclusion, our study revealed that differences in abundance of metabolites linked to carbohydrate and amino acid metabolism are maintained in *B. vosnesenskii* offspring that differ in cold tolerance, even when they have not been exposed to cold. Additionally, several exogenous metabolites associated with diet processing were differentiated across the two populations. While the patterns revealed in this study may have been influenced by colony-level differences since samples were selected from single colonies of each population, previous studies have found little among-colony differences in temperature responses for *B. vosnesenskii* (Pimsler et al., 2020) or *Bombus impatiens* (Oyen and Dillon, 2018). We therefore elected to generate segment-specific profiles given limitations of the number of samples that could be completed in a single instrument run. By characterizing metabolomes of common garden-reared bumble bee bees that differ in CT_{min} (Pimsler et al., 2020) through untargeted LC-MS/MS, our findings provide evidence that population differences in thermal tolerance may reflect, in part, constitutive differences in cellular metabolites. Assessing shifts in metabolites of these populations when challenged by cold temperatures would further reveal the significance of these conserved differences, particularly given pronounced differences in gene expression after cold exposure (Pimsler et al., 2020). Lastly, characterizing the metabolome from an additional generation of offspring from queens reared in common garden conditions (and reared by queens caught from disparate populations experiencing different local climates) would reveal if such

constitutive patterns are epigenetic or preserved across generations.

CRediT authorship contribution statement

Ellen C. Keaveny: Data curation, Formal analysis, Software, Validation, Visualization, Writing – original draft, Writing – review & editing. **Mitchell R. Helling:** Data curation, Formal analysis, Investigation, Methodology, Software, Validation, Visualization, Writing – original draft, Writing – review & editing. **Franco Basile:** Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Project administration, Resources, Software, Supervision, Validation, Writing – review & editing. **James P. Strange:** Conceptualization, Funding acquisition. **Jeffrey D. Lozier:** Conceptualization, Funding acquisition, Writing – review & editing. **Michael E. Dillon:** Conceptualization, Data curation, Formal analysis, Funding acquisition, Investigation, Project administration, Resources, Software, Supervision, Validation, Visualization, Writing – review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

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