Fuels for winter: the role of proline in overwintering bumblebee queens (*Bombus impatiens*)

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ADP/ATP - Adenosine di(tri)phosphate

AMA - Antimycin A

$CHCl_3$ - Chloroform

CoA-SH - CoA with a thiol group

CS - Citrate synthase

DI - Dionize

DTNB - 5,5′-Dithiobis-(2-nitrobenzoic acid)

DTT - Dithiothreitol

EDTA - Ethylenediaminetetraacetic

EGTA - Ethylene glycol-bis(2-aminoethyl ether)-N,N,N′,N′-tetraacetic acid

EtOH - Ethanol

ETS - Electron transport system

GDH - glutamate dehydrogenase

HCl - Hydrochloric acid

HEPES - 4-(2-Hydroxyethyl)piperazine-1-ethanesulfonic acid

HK - Hexokinase

HOAD - Hydroxyacyl-CoA dehydrogenase

MeOH - Methanol

MES - Morpholino ethanesulfonic acid

MIR05 - Mitochondrial respiration medium

Mna - malonate

NaCl - Sodium chloride

NADP - Nicotinamide adenine dinucleotide phosphate

NADH - Nicotinamide adenine dinucleotide

NAD-IDH - NAD-isocitrate dehydrogenase

Na_2SO_4 - Sodium sulfate

O-AB - O-aminobenzaldehyde

PC - Palmitoyl-carnitine

P5C - Δ1-pyrroline-5-carboxylate

PM - Pyruvate + Malate

PMD - Pyruvate + Malate + ADP

Pro - Proline

ProDH - Proline dehydrogenase

R - Routine respiration

Rot - Rotenone

Succ - Succinate

TNB - 5-thio-2-nitrobenzoic acid

Abstract

The common eastern bumblebee queens (*Bombus impatiens*) endure cold winter months by entering a diapausal state post-fertilization. During this overwintering period, these animals use stored energy reserves while maintaining a low metabolic rate. Bumblebees are thought to use primarily lipids to fuel this critical overwintering period, despite the fact that bee mitochondria do not appear equipped to break down this metabolic fuel. For some insects, lipids stored in the fat body can be converted to the amino acid proline, and this metabolic fuel has recently been discovered to be readily oxidized by bumblebee workers. My research, therefore, investigates the role of proline during overwintering in bumblebee queens. Using cellular respirometry, I determined the metabolic capacity of the muscle cells of queens to use various fuels, and if this capacity changes throughout overwintering. Surprisingly, the tested queens showed a much lower potential to oxidize proline than workers, and their capacity did not change during a four-month overwintering period. The metabolic properties of muscle tissue were further characterized using metabolic enzymes activity profile. These results further demonstrate the low potential for proline metabolism and the limitations of bumblebee queens' capacity to oxidize lipids. Body composition was measured to determine how the various energy stores (lipid, glycogen, protein) change during overwintering; however, no decrease in concentration was observed. Overall, this work clarifies the constraints of B. impatiens metabolism during overwintering.

Résumé

Le bourdon fébrile (*Bombus impatiens*) tolère les durs mois d'hiver en entrant dans un état de diapause après la fertilisation. Pendant cette diapause hivernale, ces animaux utilisent les réserves accumulées tout en maintenant un taux métabolique très bas. Les bourdons sont considérés comme utilisateurs de lipides comme carburant pendant la diapause hivernale, bien que leurs mitochondries semblent incapables de métaboliser ces molécules. Pour certains insectes, les lipides emmagasinés dans le corps gras peuvent être convertis en l'acide aminé proline, et il a récemment été démontré que ce carburant métabolique puisse être oxydé chez les ouvrières du bourdon. Ma recherche se concentre sur le rôle de la proline au cours de la diapause hivernale chez les reines du bourdon. En utilisant des techniques de respirométrie cellulaire, j'ai déterminé les capacités métaboliques des cellules musculaires des reines à utiliser multiples carburants et si ces capacités changent au cours de la diapause hivernale. Étonnamment, les reines testées ont démontré un potentiel beaucoup plus faible d'oxyder la proline que les ouvrières et leur capacité n'a pas changé au cours de la période de diapause de quatre mois. Les propriétés métaboliques du tissu musculaire ont été davantage caractérisées utilisant le profil d'activité d'enzymatique. Ces résultats démontrent en outre le faible potentiel des bourdons pour métaboliser la proline et leurs limitations à oxyder les lipides. La composition corporelle a été mesurée pour déterminer comment les différentes réserves d'énergie (lipide, glycogène, protéines) changent pendant la diapause hivernale, mais aucune diminution de concentration n'a été observée. Dans l'ensemble, ce travail clarifie les contraintes du métabolisme des bourdon pendant la diapause hivernale.

Introduction

Overview

Bees living in North America, such as the common eastern bumblebee (*Bombus impatiens*), experience harsh winters and possess adaptations to survive these challenging conditions. *B. impatiens* colonies are annual, and only young queens, which emerge in the fall, can survive the winter by burrowing in the soil for six to nine months (Szabo and Pengelly 1973; Alford 1969). Since colony foundations occurs in the spring, the bumblebee population depends on the success of the queens to survive winter. The period where the bumblebee queens are inactive and sheltered underground is very much alike hibernation for mammals. Hibernation implies body temperatures to decrease with ambient temperature and metabolism to slow down for long periods at a time (Storey and Storey 2004; Staples 2014). In the case of insects, the most appropriate term to refer to the state of dormancy they enter during winter is overwintering. As a form of diapause, overwintering is a survival strategy for insects that entails development arrest and metabolic suppression cued by ambient temperature decrease (Denlinger 2002). During this dynamic process, insect metabolism will change to meet a different set of energetic demands as it enters, maintains, and terminates diapause (Kostál 2005). An organism can suppress its metabolic activity by slowing down or stopping cellular processes that are not essential during the overwintering period such as growth, reproduction, and maintenance of unnecessary tissues such as gut or flight muscle (Hahn and Denlinger 2011). Metabolic depression allows diapausing individuals to save on metabolic cost and therefore conserves their energy reserves for longer.

Like most overwintering insects, bumblebees will use stored fuel reserves at a low rate, just enough to perform basic cellular maintenance such as protein turnover, cellular membrane

remodeling, and ion gradients maintenance (Hahn and Denlinger 2007; 2011). Other energetic demands for overwintering could include the synthesis of polyol cryoprotectants (Storey and Storey 2012) and repair associated with winter-related damages (Sinclair 2015). Bumblebees burrow underground, and the temperatures they are exposed to can vary (Alford 1969). It is still unclear what is the strategy of *B. impatiens* to endure freezing temperatures and whether they use cryoprotectants to avoid freezing (freeze avoidant) or can withstand the extracellular formation of ice (freeze-tolerant) (Sinclair 2015). To this day, the source of energy fueling these metabolic processes remains unknown for *B. impatiens*.

Energy storage and mobilization for insect diapause

When preparing for winter, insects, bumblebee queen included, must accumulate enough energy reserves to survive overwintering without feeding. Energy reserves stored in the form triglycerides in the fat body can be converted and mobilized (Arrese and Soulages 2010). Because lipids are anhydrous, they are the most energy-dense substrate utilized by animals (i.e., most energy per gram stored). This makes them ideal for slow, long-lasting activity, where their low maximal rate of ATP production is not an issue (Weber 2011) and therefore are the primary source of energy for hibernating animals (Munroe and Thomas 2004). To prepare for diapause, most insects will accumulate fat reserves (Downer and Matthews 1976), and these reserves will diminish throughout this period. This is seen in insects entering diapause at any stage of life, for example, the larvae of flesh fly, *Sarcophaga crassipalpis* entering pupal diapause possess nearly twice the lipid levels than non-diapause destined larvae and will see their lipid reserves rapidly decline at the beginning of diapause. Similarly, in blowfly larvae, *Calliphora vicina,* lipids reserves diminish to reach 20% of original content after four weeks of overwintering (Saunders

2000). In European cherry fruit fly pupae, *Rhagoletis cerasi*, lipid reserves diminish by nearly 2-fold of pre-diapause content. Of course, the diapause strategy of each species and the life stage it is in when entering diapause will shape the energetic demands of this critical period (Hahn and Denlinger 2007, 2011). Aside from needing energy reserves to fuel basal metabolism, insects entering diapause as larvae or pupae will also need sufficient reserves at the post-diapause stage for metamorphosis and tissue building (Hahn and Denlinger 2007). For the adult moth, *Caloptilia fraxinella*, there is a decrease in total lipid content after overwintering in both males and females (Andrea et *al*. 2016). Additionally, in an older study by Pullin (1987), adult nymphalid butterflies, *Aglais urticae* and *Inachis io*, have higher survival rates by the end of their overwintering period when accumulating more lipids prior. The accumulation of lipids prior to diapause or overwintering, followed by a marked depletion throughout the period, seemed to be the primary basis in all of these studies to conclude that insects use lipids as a primary fuel for overwintering metabolism. It should be said that lipids can also have functional roles for overwintering insects like the formation of antifreeze glycolipids for freeze-tolerant Alaskan beetle, *Upis ceramboides* (Walters et *al*. 2009) or egg production in the spring for *Eurosta solidaginis* (Irwin and Lee 2003).

Bumblebee queens are also thought to use primarily lipids to fuel this critical overwintering period based on the significant depletion of the fat reserve by the end of winter. Votavová et *al*. (2015), conducted microscopy analysis to evaluate adipocytes size in the fat body of *Bombus terrestris* and noted that queens before overwintering possessed larger adipocytes than queens after overwintering. The study also estimated total lipids by dissecting, drying, and weighing fat bodies. The highest amount of total lipids was again seen in queens

before overwintering and the lowest amount in queens after overwintering. In another study on *Bombus lucorum* by Vesterlund et *al*. (2014), lipids were extracted and removed for the abdomens with petroleum ether to compare abdomens weigh before and after the treatment to determine lipid content. The study concluded that warmer overwintering temperature causes queens to use more fat than queens overwintering in colder temperatures. Again, conveying the message that lipids are an essential fuel source for overwintering metabolism. Opposingly, literature shows that the bee mitochondria do not appear equipped to break down this metabolic fuel. Respirometry experiments conducted on bumblebee (*B. impatiens*) and orchid bee species flight muscle mitochondria showed that the oxygen consumption rate does not change after the addition of palmitoyl-carnitine (PC) and ADP (Teulier et *al.* 2016; Suarez et *al*. 2005). Additionally, Hydroxyacyl-CoA dehydrogenase (HOAD), an enzyme involved in fatty acid oxidation, did not display any detectable activity in orchid bee species (Suarez et *al.* 2005). Low activities of lipid catabolism enzymes were also documented in the honeybee and other bumblebee species (Crabtree and Newsholme 1972). Bumblebees' apparent low capacity to oxidize lipids conflicts with their lipid depletion during overwintering.

Fats are the predominant fuel for insect diapause. However, some insects will switch from relying on fat to using non-fat reserves, like sugars and amino acids, to meet their energetic demands (reviewed by Hahn and Denlinger 2007). Bumblebees possess carbohydrates in the form of glycogen stored in the fat body that can be converted to glucose and trehalose and transported in the hemolymph to fuel tissues during activity (Votavova et *al*. 2015; Stabler et *al*. 2015). Since glycogen has a lower energy density compared to lipids, storing enough of it to solely fuel the entire overwintering period could potentially be a challenge (Weber 2011). Insects

fat bodies also synthesize storage proteins pre-diapause. They are a source of amino acids that can be released in the hemolymph at the onset of diapause, but they are thought to be exclusively used post-diapause for tissue remodeling (Denlinger 2002; Hahn and Denlinger 2007). Whether diapause metabolism in bumblebees requires glycogen or amino acids, mobilization is unknown.

Some overwintering insects will convert carbohydrates into sugar-based cryoprotectants such as glycerol when they cannot avoid being exposed to below zero temperatures (Storey and Storey 2012). For the solitary red mason bee, *Osmia bicornis*, the dynamic and reversible process of glycogen conversion into cryoprotectants is illustrated. Fat body glycogen is significantly decreased in overwintering months when it is exposed to below zero temperatures and increases back to pre-wintering concentrations when temperature rises (Wasielewski et *al.* 2013). Amino acids can also serve as cryoprotection and be accumulated in the hemolymph of overwintering insects (Hahn and Denlinger 2007).

Proline as a metabolic fuel for insects

For some insects, lipids stored in the fat body can be converted to the amino acid proline, which can be shuttled in the hemolymph from the fat body to flight muscle mitochondria for oxidation (Bursell 1977; Weeda et *al.* 1980; Candy et *al.* 1997; Gäde and Auerswald 2002; Arrese and Soulages 2010). Proline oxidation involves a few enzyme-catalyzed steps leading its entry into the Krebs cycle. These enzymes include proline dehydrogenase, pyrroline carboxylate dehydrogenase (NADH-linked), and alanine aminotransferase (Brosemer and Veerabhadrappa 1965). When entering the mitochondria, proline is partially oxidized by the aforementioned enzymes; of proline's five carbon atoms, two are converted to carbon dioxide in the Krebs cycle

while the other three remain as alanine. The alanine also contains the nitrogen derived from proline, preventing the accumulation of nitrogenous waste products (Candy et *al*. 1997). Alanine can be reconverted back to proline in the fat body; the extra two carbon atoms required ultimately being provided by the ß-oxidation of fatty acids to acetyl-CoA (Candy et *al*. 1997). Proline can be an important source of energy when carbohydrates or lipids are not sufficient to fuel activity or cannot be directly oxidized (Arrese and Soulages 2010). There have been many studies documenting the importance of proline in insect metabolism. For the Tsetse fly, *Glossina morsitans*, proline is the main fuel utilized during flight (Bursell 1963, 1977). Proline can also have the anaplerotic role of augmenting Krebs cycle intermediates, as suggested for many insect species, including bees (Suarez et *al.* 2005). The African fruit beetle, *Pachnoda sinuata*, oxidize proline to meet energetic demand during pre-flight warm-up (Auerswald et *al*. 1999) and uses proline along with carbohydrates as metabolic fuels during flight (Zebe and Gäde 1993; Auerswald et *al.* 1999). Even *B. impatiens* workers show a potential to metabolize proline to produce ATP at a high rate. In a study from Teulier et *al*. 2016, isolated flight muscle fibres given substrates for cellular respiration respire at twice the rate when given proline. Upon more research, proline only appears to be used as a sparker for the first two minutes of the worker's flight, before trehalose oxidation takes over (Stec, 2018), meaning that despite their high capacity to oxidize it, proline does not appear to be largely used to power their flight. The amino acid would constitute an excellent energy fuel for overwintering as it does not necessitate any carrier protein to travel in the hemolymph from the fat body to the various parts of the body and as it does not produce any nitrogenous waste when oxidized (Candy et *al*. 1997).

Research questions, hypothesis, and predictions

The goal of the study was to address if 1) proline is a metabolic fuel for overwintering *B. impatiens* queens and 2) if the capacity for proline oxidation is enhanced during overwintering. In order to answer these questions, we needed to further our understanding of the metabolic capacity to oxidize different fuels for bumblebee queens. With respirometry measurements, we expected to see oxidative capacities for proline similar to the findings by Teulier et *al*. (2016), in workers of the same species. We planned to combine these observations with activities of an enzyme of proline metabolism to demonstrate the importance of proline relative to carbohydrates. We expect to observe a marked decline in lipids reserves like in the studies of Votavová et *al*. (2015) and Vesterlund et *al*. (2014) while demonstrating with respirometry and enzymatic activities that the thorax muscle mitochondria are unable to oxidize lipids directly. Along with significant proline oxidation capacities, such observation would solidify the hypothesis that proline is being synthesized from fatty acids in the fat body. This study describes the potential for carbohydrates, lipids, and amino acids usage. The mitochondrial metabolism of *B. impatiens* during overwintering was characterized by key enzyme's activities and respirometry procedures. The relative usage of energetic reserves was characterized by body composition analysis in queens randomly sampled at different overwintering periods.

Methods

Insect holding and overwintering conditions

Mature bumblebee colonies (*B. impatiens*), were purchased from BioBest Canada Ltd. (Leamington, ON). Colonies were maintained in their plastic hive containers inside cardboard boxes and fed with sugar water (1:1 v/v) *ad libitum* and pollen balls every three to four days. The room housing the colonies was kept at 25°C with 12 h:12 h light: dark photoperiods. Colonies continued to proliferate in the hive boxes, producing new queens approximately one month after delivery. New queens were collected when silver setae of newly emerged individuals were no longer present. To induce dormancy, the queens were placed in 50 ml conical plastic tubes in an upright incubator in the dark and set at 6°C. The tube lid was punctured to allow airflow, and the chamber contained a large beaker of distilled water to maintain humidity to approximatively 30%. Queens were randomly divided into five overwintering periods lasting 0, 4, 8, 12, and 16 weeks. On the exact day they had overwintered their designated periods, queens were weighed, and either muscle respiration rate measurements were conducted, or the whole queens were stored at -80°C for further biochemical assays.

Thorax muscle fibres isolation

Dormant queens were taken from the incubator, while non-overwintering queens were taken directly from the hive box. Individuals were weighed and afterward dissected to isolate the thorax. Following the methods of Teulier et *al.* (2016), muscle fibres were removed using fine forceps and transferred to an ice-cold preservation buffer BIOPS (Kuznetsov et *al.* 1998). The buffer contained 10 mM Ca- EGTA buffer, 0.1 mM free calcium, 20 mM imidazole, 20 mM taurine, 50 mM K-2-(N-morpholino) ethanesulfonic acid (MES), 0.5mM Dithiothreitol (DTT),

6.56 mM $MgCl_2$, 5.77mM ATP, 15 mM phosphocreatine, pH 7.1 at 0°C. Between 1 and 2 mg of muscle fibres were taken and dabbed on a piece of weighing paper to remove the excess buffer and weighed. Muscle fibres were then transferred to ice-cold respiration buffer MIR05 (Kuznetsov et *al.*, 1998), containing 0.5 mM EGTA, 3 mM $MgCl_2$, 60 mM K-lactobionate, 20 mM taurine, 10 mM KH_2PO_4, 20 mM HEPES, 110 mM sucrose and 1 g l^{-1} of fatty acid-free bovine serum albumin, pH 7.1 at 30°C for at least 30 minutes before respirometry measurements took place.

High-resolution respiration rate

The capacity of thoracic flight muscle fibres to oxidize various metabolic substrates was determined using high-resolution respirometry. Oxygen consumption was measured with a high-resolution respirometer (Oxygraph-2k, Oroboros® Instruments, Innsbruck, Austria) to determine mitochondrial respiration rate. Electrodes were calibrated daily, and instrument background rates were assessed each month. Quadruplicate measurements were taken for all individuals to account for variation in muscle fibre bundles respiration. The muscle fibres were placed in 2 ml of MIR05 inside sealed respirometer chambers, and oxygen was added to achieve a concentration of approximately 500 nm O_2 ml^{-1}. All assays were conducted at 37°C. Substrates and inhibitors were added to the respirometry chambers with Hamilton syringes and concentrations were based on saturating levels previously established by Teulier et *al.*, 2016. The respiration rates of permeabilized muscle fibres were measured following the addition of the conventional substrates activating the different complexes of the ETS. Complex I was stimulated with pyruvate (5 mM) and malate (2 mM), followed by ADP (2.5 mM). Complex II substrate, succinate (10 mM), was then added, followed by proline (10mM). Complexes I, II, and III were then inhibited

sequentially using rotenone (0.5 µM), malonic acid (15 mM), and antimycin A (2.5 µM) to determine respiration rates independent of each inhibited complex. A representative trace of the change in fibre respiration rate according to substrate and inhibitor addition is depicted in Figure 1. The capacity of the muscle fibres to oxidize fatty acids was also tested with another respirometry protocol consisting of sequentially adding malate (2 mM), PC (0.1 mM), and ADP (2.5 mM). Fatty acid derivative, PC, was selected as it is known to be transported into the mitochondrial matrix and oxidized in insects (Hansford and Johnson 1976; Childress et *al*. 1966; Chamberlin 1987). Pyruvate (5 mM) was further added to confirm that the muscle fibres were able to respond to the activation of complex I.

Enzyme assays

Bumblebee queens were dissected, and the thorax and abdomen were weighed separately. Manipulations from this point forward were executed on ice. The abdomen was minced with scissors and combined in 9 volumes of cold homogenization buffer (50 mM Imidazole, 1 mM ethylenediaminetetraacetic (EDTA), 2 mM $MgCl_2$, pH 7.4 at 4°C, 5 mM DTT and 0.5% Triton X-100). Each thorax was also minced in 9 volumes of homogenization buffer, but DTT and Triton X-100 were omitted as they largely decrease the activity of proline dehydrogenase (ProDH). The samples were homogenized at 10,000 rpm three times for 10 seconds between 30-second rest intervals. A 300 µl aliquot of thorax homogenate was set aside for ProDH measurements and centrifuged for 5 minutes at 2400g at 4°C. The supernatant was tested for ProDH activity. The rest of the thorax homogenate was combined with an equal volume of homogenization buffer with 10mM DTT and 1% Triton X-100 to match the desired final concentrations (5mM DTT and 0.5% Triton X-100). Homogenates were sonicated three times for

10 seconds between 30-second rest intervals and centrifuged for 5 minutes at 2400g at 4°C. The thorax supernatant containing DTT and Triton X-100 was tested for hexokinase (HK) and citrate synthase (CS) activity. The abdomen supernatant was tested for β-hydroxyacyl-CoA dehydrogenase (HOAD) activity.

Enzymatic activities of HK, HOAD, CS, and ProDH were measured with a plate spectrophotometer (Synergy 2, Biotek Instruments, Winooski, VT, USA) at 37°C. The activities of HK and HOAD were measured through the appearance and disappearance, respectively, of NADH at 340 nm. The activity of citrate synthase was determined through the production of TNB, a product of the colorimetric reaction between DTNB and CoA-SH, which was monitored by measuring the increase in absorbance at 412 nm. The activity of ProDH was measured by the rate of appearance of Δ1-pyrroline-5-carboxylate (P5C) by way of its colorimetric reaction with o-aminobenzaldehyde (O-AB) at 440 nm. The assay conditions to achieve Vmax were as follows: **HK**: 100 mM Tris-imidazole (pH 8.1), 10 mM KCl, 10 mM $MgCl_2$, 5 mM D-glucose (omitted for control), 1 mM NADP, 5 mM ATP and 1.25 U/250 µl glucose-6-phosphate dehydrogenase. **HOAD:** 100 mM Tris-HCl (pH 7.0), 5mM EDTA, 0.1 mM acetoacteyl-CoA (omitted for control), 0.3 mM NADH. **CS**: 50 mM Tris-HCl (pH 7.4), 0.3 mM acetyl-CoA, 0.5 mM oxaloacetate (omitted for control) and 0.1 mM DTNB. **ProDH**: 100 mM potassium phosphate (pH 7.2), 0.12mg ml^{-1} o-aminobenzaldehyde (O-AB), 0.012 mg ml^{-1} cytochrome c, 50 mM proline. The reaction was stopped after 15 minutes by the addition of 10mg ml^{-1} of O-AB in 6 N HCl.

Extraction and colorimetric assay of lipid, protein, glycogen and free carbohydrates

The protocol for the extraction of body reserves was adapted from Lorenz, 2003. Each abdomen was taken from storage in the -80°C freezer, weighed, and transferred to a 50 ml glass vial and homogenized (OMNI International, Kennesaw, GA, USA) with 1 ml of Na_2SO_4 and 1.5 ml of MeOH. The homogenizer pestle was then rinsed with 3 ml of $CHCl_3$: MeOH (1:1, v/v) directly into the glass vial. A subsample of 1 ml of the homogenate was collected and transferred to a 2 ml centrifuge tube. The remaining homogenates were stored at -80°C and later used for dry weight determination. The homogenates subsamples were then sonicated for 5 minutes (Sonics & Materials, Inc., Newtown, CT, USA) and centrifuged (Sorvall Legend Micro 21R, Thermo Scientific, Osterode, Germany). All centrifugations were performed at 21 000 g for 10 minutes at 4°C. The supernatant was transferred into a clean centrifuge tube, and the pellet was sonicated and centrifuged again with 300 µl of $CHCl_3$: MeOH (1:1, v/v), ensuring complete extraction of lipids and carbohydrates. The second resulting supernatant was combined with the first and set aside. The pellet was dried completely on a thermoblock set at 99°C before adding 500 µl of Na_2SO_4 saturated 66% EtOH. The pellet was left to stand for 10 minutes in order to dissolve the remaining glucose. Following this, the pellet was vortexed, sonicated and vortexed again. Afterward, the dissolved pellet was centrifuged to remove and discard the supernatant. The new resulting pellet was dried completely on a thermoblock at 99°C. To the dry pellet, 200 µl of 10% KOH was added before being vortexed, sonicated, and left to macerate for 30 minutes at 99°C while vortexing every 10 minutes. An aliquot was taken and diluted 1:3 (v/v) in DI water for determination of protein. To the remaining dissolved pellet, 150 µl of 95% EtOH was added. The pellet was left standing for 10 minutes, then centrifuged. The supernatant was discarded, and the pellet dried completely on a thermoblock at 99°C. To break down glycogen into glucose, 400 µl

of DI water at 99°C was added. This portion was later used to determine glycogen. To the combined supernatant, 500 µl of $CHCl_3$ and 300 µl of NaCl 1M were added, and the sample was vortexed and centrifuged, resulting in an aqueous epiphase and an organic hypophase. The aqueous epiphase was dried in a Speed-Vac and redissolved in 500 µl of DI water for determination of free carbohydrate. The organic hypophase was dried in a fume hood overnight and redissolved in 1000 µl of hexane and 500 µl of NaCl 1M. The sample was vortexed and centrifuged, and the lipid portion dissolved in hexane was taken for determination of lipid.

To determine the concentration of lipid, protein, glycogen and free carbohydrates in the abdomen of bumblebee queens, colorimetric assays were conducted in triplicates using a plate spectrophotometer (Synergy 2, Biotek Instruments, Winooski, VT, USA). The protein assay was performed with a bicinchoninic acid protein assay kit (Sigma- Aldrich) with a bovine serum albumin protein standard. Determination of glycogen and free carbohydrates was performed with the anthrone method (Lorenz 2003; Kaufmann and Brown 2008). The anthrone reagent was made with 1.4 mg ml^{-1} anthrone in 95-98% sulfuric acid and DI water to achieve a 28% - 72% water: acid ratio. A glucose standard curve ranging from 0 µg $µl^{-1}$ to 0.04 µg $µl^{-1}$ was made from anhydrous glucose in DI water and anthrone reagent (ratio of 1:25, v/v). A subsample of 10µl of both the glucose and glycogen extractions was added to 240µl of anthrone reagent and heated with the standard in the same well plate for 17 minutes at 99°C. After cooling for 5 minutes, the absorbance was read at 625 nm. Lipid concentration was determined using the phospho-vanillin method (Lorenz 2003). Vanillin- phosphoric reagent was made with 6 mg ml^{-1} of vanillin in hot DI water and phosphoric acid 85% to achieve a 20% -80% water: acid ratio. A standard was made from soybean oil in hexane (1 mg per 1 ml). This standard (0, 50, 100, 200, and 400 µl)

and a portion of the lipid extraction (30, 75, and 100 µl) were pipetted into glass vials. The standard and lipid portions were placed in a water bath at 99°C. Once all of the solvent was evaporated, 200µl of sulfuric acid (95%-98%) was added to each glass vials and heated in the water bath for 10 minutes. The vanillin reagent was then added to the 5 ml level, and the glass vials were removed from heat and briefly mixed. The absorbance was read at 625 nm. For body reserve concentrations, values were obtained by comparing absorbance values to the standard curve for lipids, protein, glycogen, and free carbohydrates. Concentrations were first expressed in µg of reserve per mg of fresh abdomen weight, then divided by the percentage of dry mass to be expressed as µg of reserve per mg of dry abdomen weight.

Determination of dry weight

To account for possible water loss while overwintering, the dry weight of the queen abdomens assayed for body composition was determined for the purpose of distinguishing changes in body composition independent of water loss. A subsample of 2 ml of the crude homogenate was transferred in a pre-weighted aluminum container and put in a fume hood for the organic solvent to evaporate and then put in an oven at 60°C for 24 hours. The dry homogenate was weighed to identify the percentage of dry mass in each abdomen.

Data Analysis

All data analyses were conducted using the SIGMAPLOT V.12.5 and SYSTAT V.12 software. Analyses of variance (ANOVA) were conducted to determine if energy stores and mean wet and dry abdomen mass change throughout overwintering. Changes in total body mass throughout overwintering was described by a linear regression using fresh mass data of

individuals collected randomly every two weeks from 0 to 16 weeks. All data were tested for normality (Shapiro-Wilk test) and homogeneity (Levene's test). For the data that were not normally distributed, log transformations were performed. For protein concentrations, data was still non-normal after log transformation, so a non-parametric test was used (Kruskal-Wallis ANOVA on ranks). For lipid concentrations, the ANOVA test was performed with pairwise comparison (Turkey test) to detect which periods were significantly different from each other. For CS activity, the data were normally distributed but showed heteroscedasticity; thus, a post-hoc test for unequal variance (ANOVA with Games-Howell post-hoc test) was conducted. Muscle fibre respiration rates (pmol O_2 s^{-1} mg^{-1}) were tested for differences associated with substrates and inhibitors addition, and for differences between overwintering periods using a two-way ANOVA with repeated measures. The test was performed with pairwise comparison (Turkey test) and with adjustments for multiple comparisons (Holm-Sidak method).

Results

Mitochondrial energy metabolism does not change during overwintering

The capacity of *B. impatiens* flight muscle mitochondria to oxidize various substrates does not change during the progression of a 4-month overwintering period (Fig. 2) (overwintering period: $F_{4,66} = 0.701$, $p = 0.607$; substrate: $F_{6,66} = 282.016$, $p < 0.001$; overwintering period x substrate: $F_{24,66} = 0.683$, $p = 0.850$). The overwintering period does not affect the mitochondrial respiration rate following the addition of substrates and inhibitors. Following the addition of the complex I associated substrates pyruvate and malate, ADP increased mitochondrial respiration rate by 10-fold ($p < 0.001$). The addition complex II substrate, succinate, further increased the oxygen consumption by 1.2-fold ($p < 0.001$). Proline, however, did not significantly increase the respiration rate after the addition of succinate (from 409.68 ± 19.19 to 432.81 ± 19.75 pmol O2 s^{-1} mg^{-1}; $p = 0.239$). Though not statistically significant, the response to the addition of proline was detectable for most samples, causing a slight but noticeable increase in oxygen consumption trace (Fig. 1).

The addition of inhibitors allowed us to further determine mitochondrial respiration associated with individual complexes in *B. impatiens*. The addition of the complex I inhibitor, rotenone, significantly decreased oxygen consumption by 60% ($p < 0.001$). Complex II inhibitor, malonate, further decreases oxygen consumption ($p < 0.001$) to match the rate of respiration observed before the addition of ADP ($p = 0.223$), suggesting that the rest of the respiration was inhibited with complex II. The addition of complex III inhibitor, antimycin A, did not significantly decrease oxygen consumption ($p = 0.151$).

Additionally, the activity of enzymes involved in proline (ProDH) and carbohydrate (HK) break-down demonstrated a high capacity for hexose sugar phosphorylation (41.71 ± 1.14 U g^{-1}) and a much lower activity for proline dehydrogenase (1.71 ± 0.07 U g^{-1}) in all of our sampled queens (Fig. 3). Throughout overwintering, bumblebee queens showed no change in HK activity ($F_{4,39} = 2.102$, $p = 0.099$). For ProDH, there was a significant increase of activity at 12 weeks (2.23 ± 0.12 U g^{-1}) compared with non-overwintering queens (1.52 ± 0.11 U g^{-1}) (Fig. 3D $F_{4,39} = 6.741$, $p < 0.001$). The activity of CS did not change during the progression of overwintering, except in bumblebee queens sampled at 16 weeks of overwintering where there was a significantly higher CS activity (102.41 ± 4.33 U g^{-1}) versus queens sampled before overwintering (67.71 ± 7.35 U g^{-1}) (Fig. 3B $p = 0.010$).

Fatty acids are not oxidized in mitochondria

The ability of bumblebee queens to oxidize fatty acids was tested with PC as a substrate (Fig. 4). The respiration rates resulting from each substrate addition are similar across the tested overwintering time periods (overwintering period: $F_{4,33} = 1.434$, $p = 0.287$; substrate: $F_{3,33} = 65.370$, $p < 0.001$; overwintering period x substrate: $F_{12,33} = 1.085$, $p = 0.403$). The addition of PC followed by ADP did not increase oxygen consumption in bumblebee queen mitochondria ($p = 0.133$). Furthermore, the activity of HOAD in bumblebee queens showed low activity (1.69 ± 0.16 U g^{-1}) overall and no significant increase throughout overwintering ($p = 0.147$) (Fig. 3C).

Body composition does not change during overwintering

The bumblebee queens used in our experiment decreased in body mass during the progression of the 16 weeks overwintering period ($r_2 = 0.181$, $p < 0.001$, Mass= -8.18 (overwintering period) + 542.61) (Fig. 5). Furthermore, fresh mass of abdomens from non-overwintering queens were significantly heavier than of queens who endured overwintering for 12 and 16 weeks (Fig. 6A) ($F_{4,25} = 4.545$, $p < 0.007$). This significant difference was lost when testing for differences in abdomen dry mass ($F_{4,25} = 1.160$, $p = 0.352$)(Fig. 6B).

For the entire overwintering period we observed in our bumblebee queens, we could not detect any significant decrease in glycogen ($F_{4,25} = 0.224$, $p = 0.922$) or protein ($x_2 = 4.052$; *d.f.* = 4, $p = 0.399$) concentration amongst abdomen (Fig. 7). The four weeks period had a higher variance due to one individual with 15 times more glycogen than other queens sampled. There was a significant effect of the overwintering period on the free carbohydrate concentration in the abdomen ($F_{4,25} = 2.803$, $p = 0.047$); however, the pairwise analysis did not identify where this difference occurred. Only in lipid concentration, did we detect that queens from week 8 and 16 were significantly different from each other ($F_{4,25} = 3.000$, $p = 0.038$).

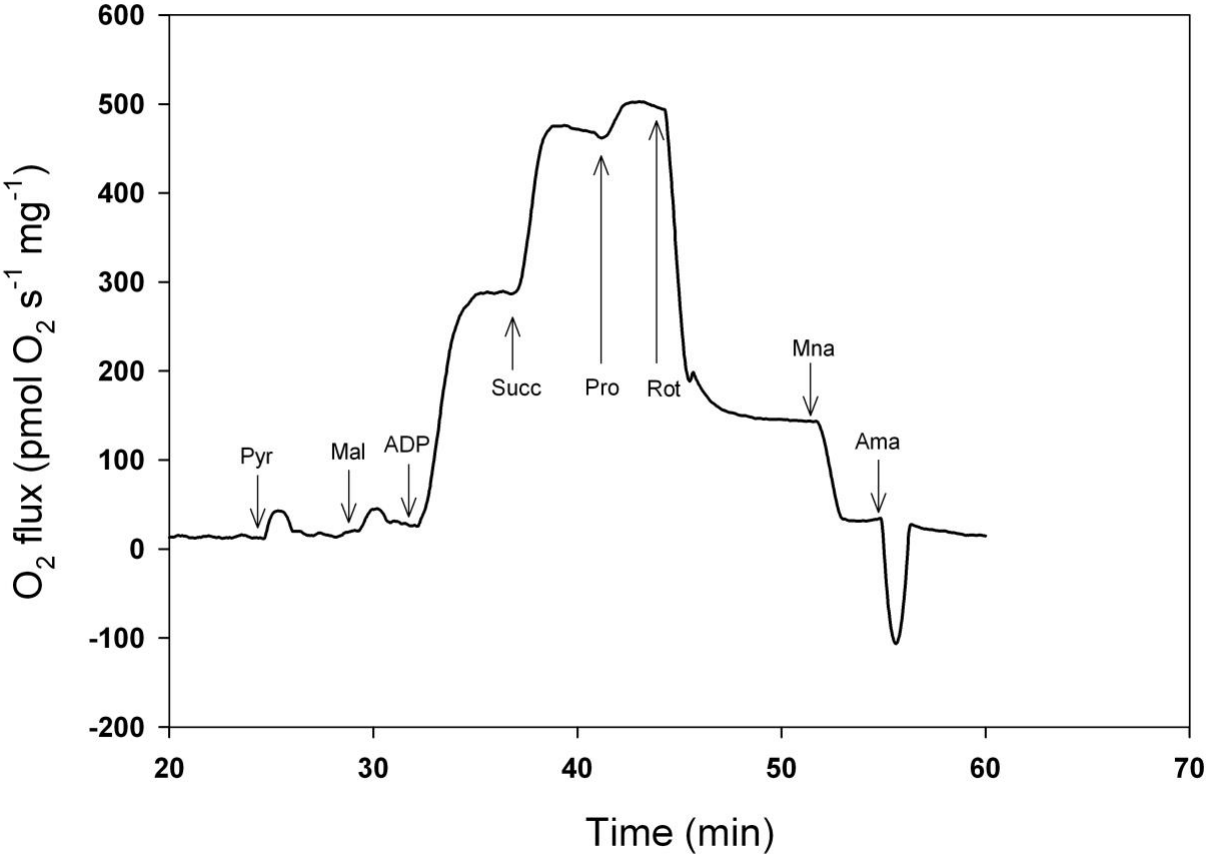

Figure 1 Traces of oxygen flux (pmol O2 s^{-1} mg^{-1}) from thoracic muscle fibres of a B. impatiens queen after addition of metabolic substrate: pyruvate (Pyr), malate (Mal), ADP, succinate (succ), proline (Pro) and inhibitors: rotenone (Rot), malonate (Mna), and antimycin A (Ama).

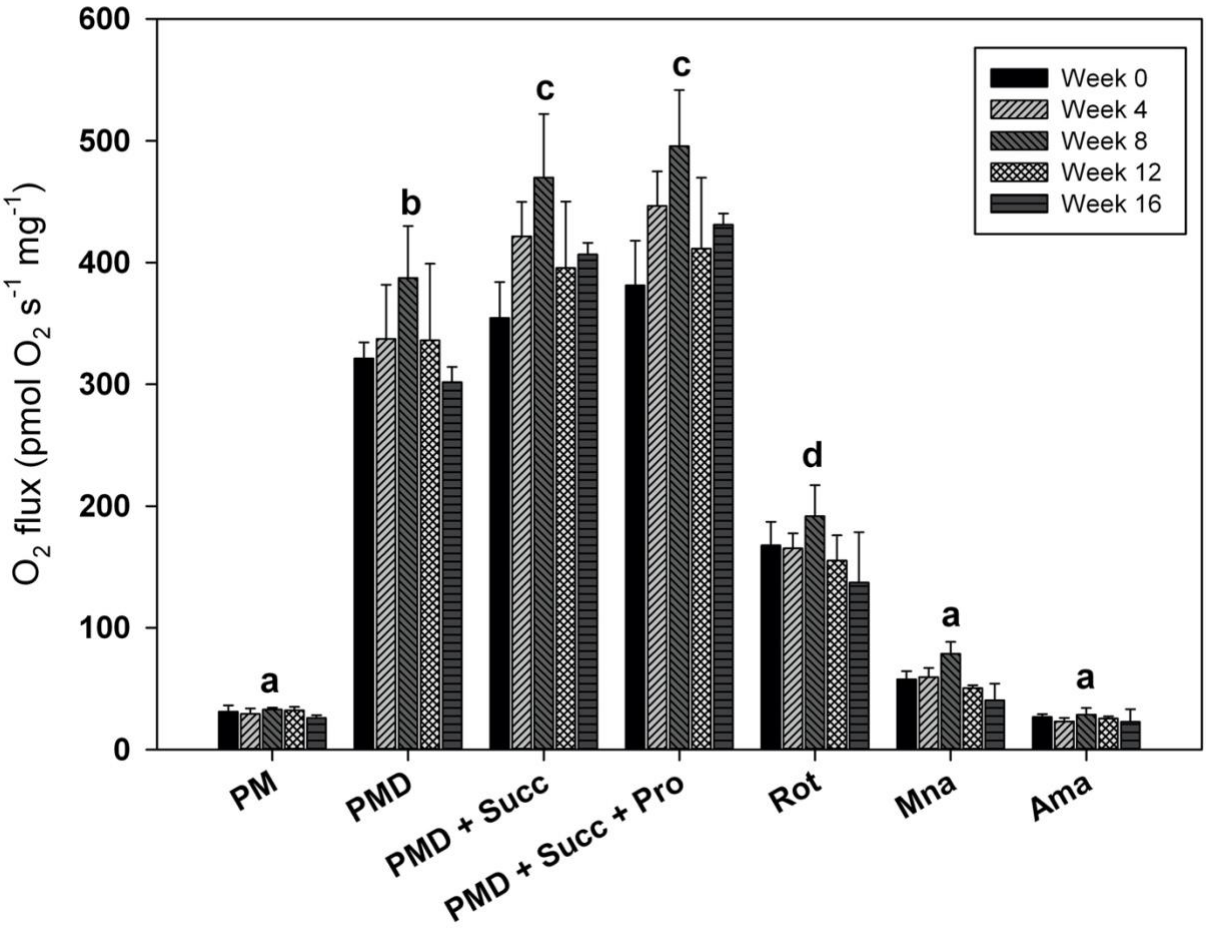

Figure 2 Respiration rates (pmol O2 s^{-1} mg^{-1}) of thoracic muscle fibres of B. impatiens queens over the progression of the overwintering time periods. Data are means ± SE of 3-4 (except at week 16 where n=2) queens sampled for every period for a total of 16 queens. The oxygen fluxes recording begins with the addition of pyruvate + malate (PM), followed by ADP (PMD), succinate (SUCC), proline (PRO), rotenone (ROT), malonate (MNA), and antimycin A (AMA). There is no difference among overwintering groups within substrates or inhibitors ($p = 0.607$). Statistical difference ($p < 0.05$) between substrates and inhibitor are indicated with different letters.

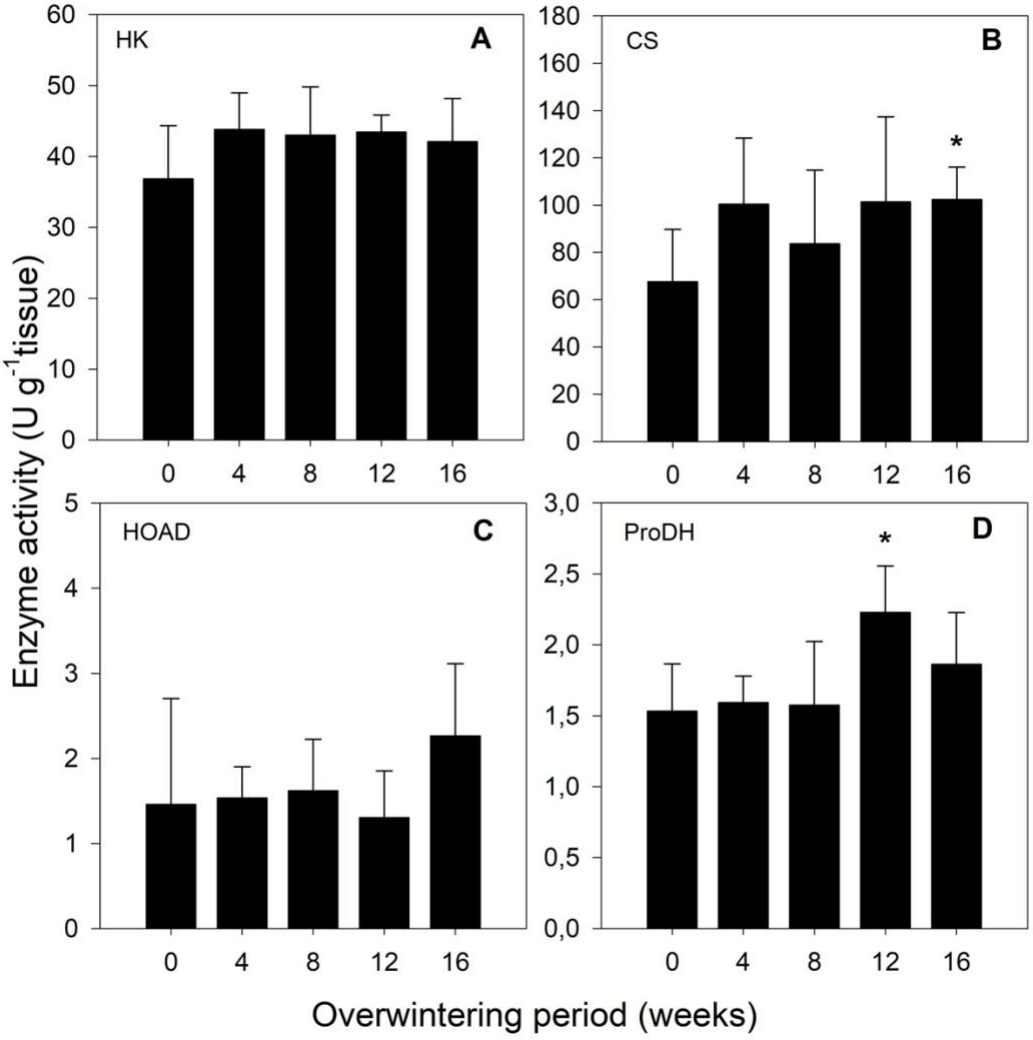

Figure 3 Enzyme activities of HK (A), CS (B), and ProDH (D) in the thorax and HOAD (C) in the abdomen of *Bombus impatiens* queens at different overwintering periods. Activities are represented in U per gram of tissue where U is 1 mmol of substrate per minute. Data are means ± SE of 5-10 queens sampled for every period for a total of 30 queens for HOAD assay and 44 queens for the other assays. Overwintering groups with activities of enzyme statistically different form non-overwintering queens (0) are represented with asterisks.

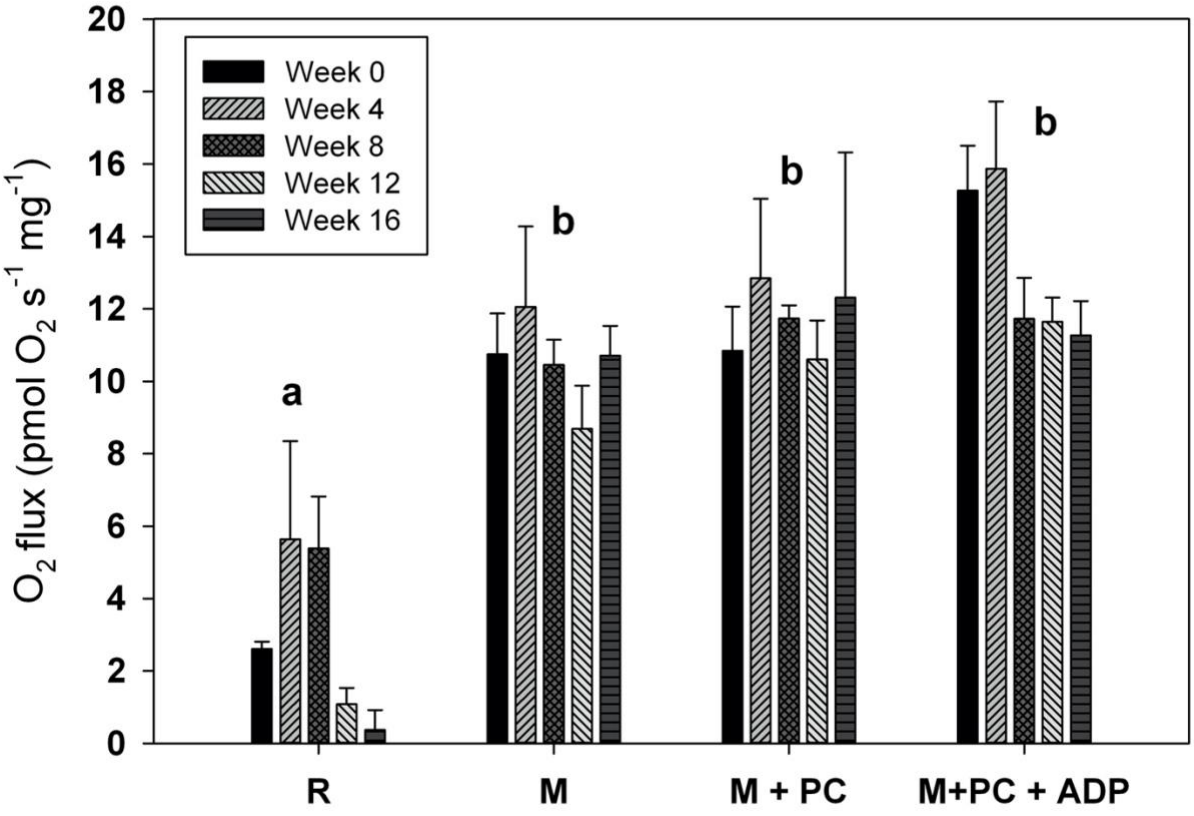

Figure 4 Effects of palmityol-carnitine on respiration rates (pmol O_2 s^{-1} mg^{-1}) measured on thoracic muscle fibres of *B. impatiens* queens at different overwintering period (n= 2-4). R: routine respiration, M: malate, PC: palmitoyl-carnitine. Statistical difference ($p < 0.05$) between substrates are indicated with different letters. There is no difference among overwintering groups within substrates ($p = 0.403$).

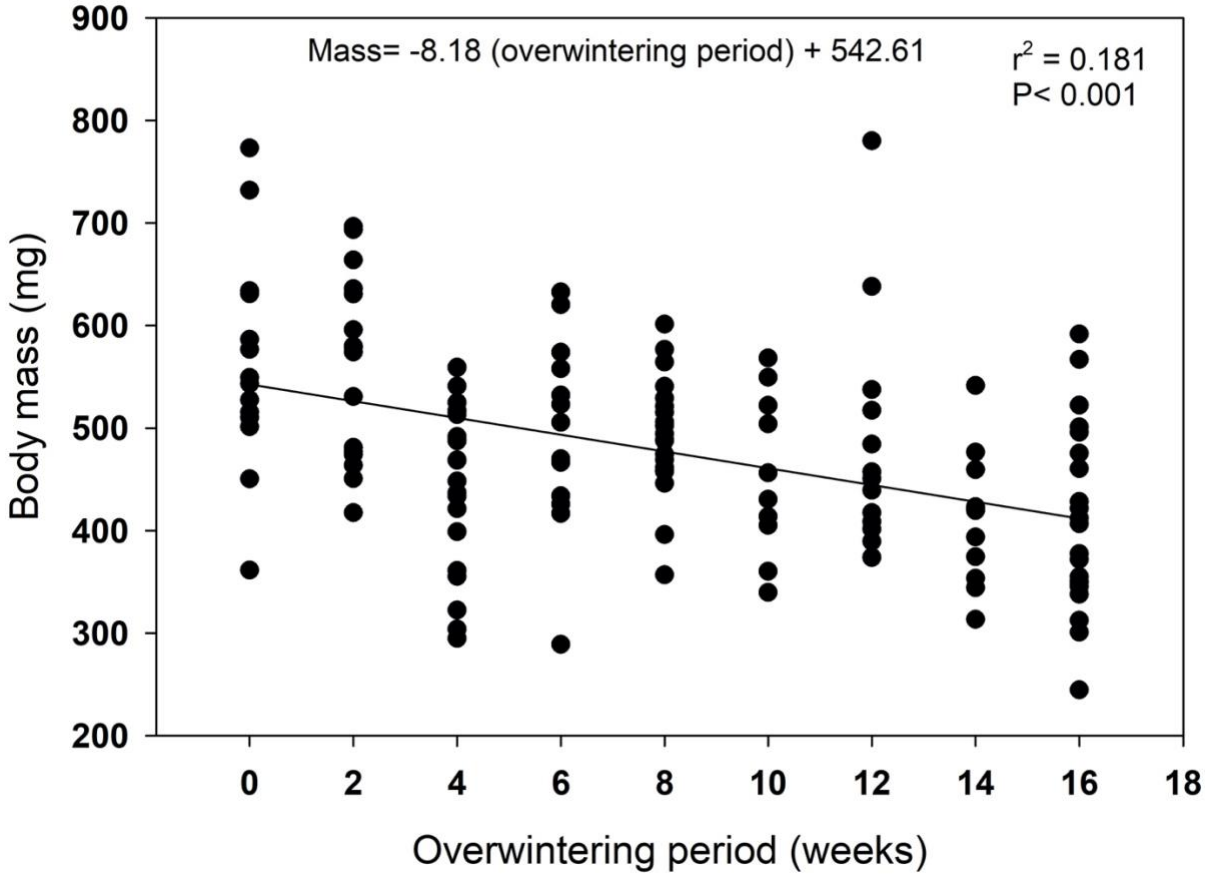

Figure 5 Regression analysis of total fresh body mass (mg) of *B. impatiens* queens after weeks of overwintering. Between 10 and 21 individuals are represented per period for a total of 141 individuals. Regression line equation is shown on the graph.

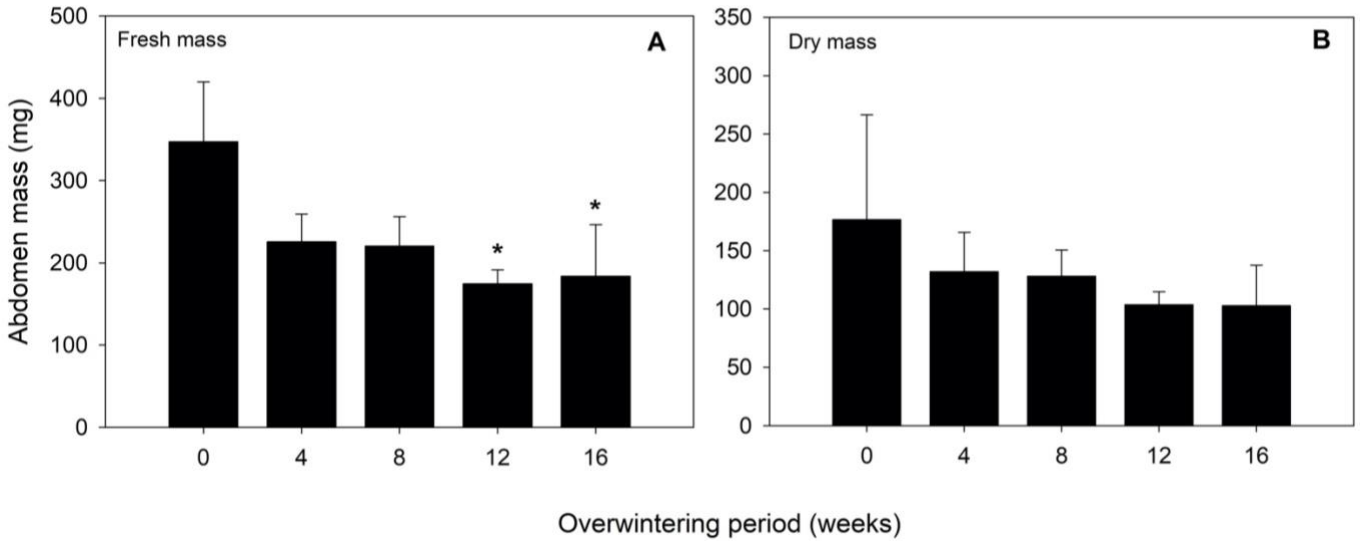

Figure 6 Fresh (A) and dry (B) abdomen mass (mg) of *Bombus impatiens* queens after weeks of overwintering. Every week is represented by 6 individuals for a total of 30 individuals. Bars statistically different form non-overwintering queens (0) are represented with asterisks.

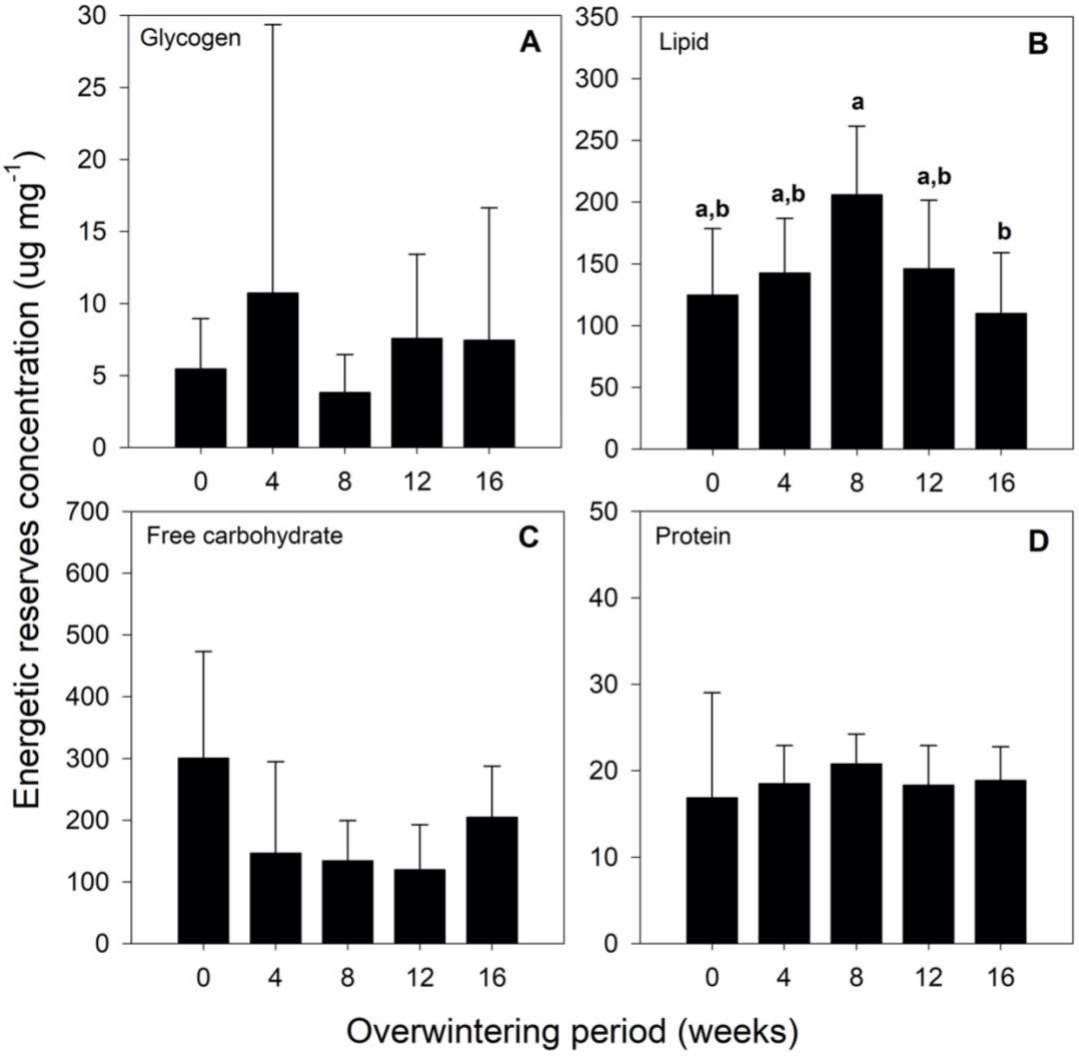

Figure 7 Profile of glycogen, lipid, free carbohydrate, and protein per mass of dry whole abdomen of *B. impatiens* queens at different overwintering periods. Data are means of ± SE of 6 queens sampled for every period for a total of 30 individuals. Statistical difference ($p < 0.05$) between energetic reserves concentrations are indicated with different letters.

Discussion

Mitochondrial properties throughout overwintering

Insect diapause is a dynamic process, and that implies a metabolic depression beyond the passive thermal effect (Muleme et *al*. 2006; Kostal 2006). During overwintering, the degree of metabolic suppression varies among insects (Hahn and Denlinger 2007). The metabolic rate will decrease in response to ambient temperatures, and this dependence is described with Q_{10} values, ranging from 1.5 to 3 (Tauber et *al*. 1986). Insect diapause involves shutting down or greatly decreasing the activity of energetically expensive biochemical and physiological systems (Tauber et *al*. 1986; Hahn and Denlinger 2007) to actively suppress respiration aside from temperature-related suppression. Bumblebees queens are known to enter diapause (Alford 1969; Owen et *al*. 2013; Amsalem et *al*. 2015), but whether they experience such a hypometabolic state during overwintering is not reported. Metabolic depression reduces the need for mitochondrial activity. The suppression of mitochondrial metabolism in animals can come from a reduction in the abundance of mitochondria or in the amount of enzyme protein per mitochondrion, or from reversible post-transcriptional regulatory controls that inhibit mitochondrial functions (McMullen and Storey 2008; Storey and Storey 2012).

The mitochondrial content of flight muscle does not change for *B. impatiens* queens throughout our experimental overwintering period. Using two indicators of mitochondrial respiratory capacity, muscle fibre respiration, and the activity of the mitochondrial enzyme CS, bumblebee queens do not reduce tissue metabolic capacity during overwintering. Changes in mitochondrial capacity during overwintering in insects are not well documented, although McMullen and Storey (2008) suggested that measuring mitochondrial DNA levels or

mitochondrial enzymes activities would appropriately monitor changes in mitochondrial content. In a study exploring how short-term cold exposure (4°C) influences mitochondrial respiration in the tropical cockroach, *Gromphadorhina coquereliana*, changes in mitochondrial respiration in response to temperature were detected (Chowanski et *al*. 2017). A significant decrease of 35 % in state 3 respiration was observed in fat body mitochondria following a three-hour exposure to cold conditions, whereas muscle mitochondria showed no changes in oxygen consumption rate. Similarly to our approach, the measurement of oxygen consumption by mitochondria was conducted at a standard temperature of 25°C encountered during activity. In a mammalian hibernator, the 13-lined ground squirrel, state-3 respiration rate (measured at 37°C) was 1.5-fold lower in skeletal and cardiac muscle and threefold lower in liver tissue compared to the summer active state (Muleme et *al*. 2006; Staples 2014). Reduced potential for mitochondrial metabolism has been demonstrated for various ectotherms during metabolic depression with mitochondrial enzymes activities. Both the larvae of the freeze-tolerant moth, *E. solidaginis,* and the freeze avoidant moth, *Epiblema scudderiana* had their activities of CS, NAD-isocitrate dehydrogenase (NAD-IDH) and glutamate dehydrogenase (GDH) reduced by half in winter months compared to values in the fall (Joanisse and Storey 1994). This response is also observed in aquatic arthropods such as the Antarctic krill, *Euphausia serperba*. Females have lower CS activity during winter when food sources become scarce, and the organism has to overwinter (Cullen et *al*. 2003). For hibernating amphibian, the common frog, *Rana temporaria*, metabolic depression is also associated with the significant decrease in CS and cytochrome c oxidase (CCO) activities (Boutilier and St-Pierre 2002). The absence of a reduction in CS activity indicates that the abundance of mitochondria is not reduced during overwintering for *B. impatiens* queens. So not

only do overwintering queens' mitochondria appear capable of oxidizing substrates at the same rate as non-overwintering queens, they do not seem to undergo mitochondrial degradation.

The properties of mitochondrial ETS are conserved throughout the overwintering period. The addition of substrate, sequentially activating the ETS complexes, results in a similar response in oxygen flux for individuals of all overwintering periods. Because both hibernation and diapause are metabolically dynamic states, they may involve shifts from one energy source to another (Hahn and Denlinger 2010; Muleme et *al*. 2006). Based on our hypothesis, such shift in bumblebee metabolism energy source was expected to be from carbohydrates oxidation to proline oxidation. This would imply a significant contribution of proline dehydrogenase in cellular respiration, hence a difference in complex I-independent respiration between non-overwintering and overwintering queens. In mammalian hibernators, such differential regulation of ETS complexes has been shown. Mitochondrial respiration is similar between hibernating and non-hibernating animals when using pyruvate or fatty acids derivatives as substrate, but hibernators show a much-reduced capacity to use succinate as a substrate. This indicates suppression of oxidative capacity occurring downstream of complex II (Staples 2014). The mechanisms underlying this sort of mitochondrial suppression are still unknown but are hypothetically post-translational modifications of ETS proteins, although it remains to be confirmed (Staples 2014). In the case of *B. impatiens*, the capacity for the fatty acid derivative, PC, and proline oxidation was very low for non-overwintering individuals and remained the same as queens overwintered. Nevertheless, it is possible that the temperature-dependence of individual ETS complexes are different. Indeed, freeze-tolerant moth *E. Solidaginis*, mitochondrial respirometry shows that proline is the preferred substrate at 20°C and that the

importance of proline supported respiration increases when assayed at 1°C (Ballantyne and Storey 1985). It is therefore, that differences in ProDH activity may be observed at low temperatures in overwintering queens, which we did not account for. Our results do not support a shift in energy substrate choice for overwintering *B. impatiens* and do not demonstrate any biochemical inhibitions on the ETS complexes of the muscle mitochondria.

Role of proline

Our hypothesis that proline is a central metabolic fuel in overwintering queens is not supported by our results. In fact, *B. impatiens* queens exhibit a much lower capacity to oxidize proline compared with workers of the same species (Teulier et *al*. 2016). This apparent difference between castes suggests that proline is more of a substantial oxidative fuel in workers, although the functional significance of this capacity is still unknown. The low maximal activity of proline dehydrogenase also supports the marginal role played by proline during overwintering in bumblebee queens. The ProDH activities are indeed observed in a wide range of insects. A known proline user, the tsetse fly (*Glossina austeni*), has an average activity of 40 µmol min^{-1} g^{-1} while the honey bee (*Apis mellifera*), a strict carbohydrate user, has an average activity of 1.5 µmol min^{-1} g^{-1} (Crabtree and Newsholme 1970). Our recorded ProDH activities fall closer to the non-proline user, the sister species *A. mellifera*. The activity of ProDH is not well studied, to say the least, during the overwintering process in insects. This could be because the hypothesis of proline as an overwintering fuel is relatively new (Teulier et *al*. 2016; Sinclair and Marshall 2018). Proline is regularly observed to be in high quantities in overwintering insects' hemolymph in pair with trehalose, but its role has been mostly associated with its cryoprotecting effect rather than as a metabolic fuel (Misener et *al.* 2001; Rozsypal et *al.* 2013).

Role of lipids

This study demonstrates that *B. impatiens* is limited in its capacity for lipid oxidation. Respirometry measurements showed that muscle tissues of bumblebee queens are unable to oxidize PC. Our results are coincident with previous work on bee's capacity for fatty acids oxidation, using respirometry procedures and PC as a substrate (Suarez et *al*. 2005; Teulier et *al*. 2016). These properties thought to be typical of the flight muscle of a nectar-feeding, high metabolic rate organism, fueling flight solely using carbohydrates, and possibly with the help of proline to increase TCA cycle intermediates (Teulier et *al.* 2016). Even though lipid oxidation could, in theory, be sufficient to fuel a lower metabolism, such as the one overwintering bumblebee queens experience, this capacity seems to be lost in *B. impatiens*. In addition, queens had very low enzyme activity for HOAD, an enzyme involved in fatty acid oxidation, in both the abdomen and thorax and the activity did not increase during overwintering. For overwintering insects that use lipids, activities of some enzymes involved in lipid oxidation are rising throughout winter. The freeze-avoiding moth, *E. scudderiana*, shows a 31% increase in HOAD activity from September to March to reach activities three times higher than our values for HOAD in bumblebee queens (Joanisse and Storey 1996). For the freeze-tolerant moth, *E. solidaginis*, which is thought not to use lipids during overwintering, activities of HOAD decreased upon September and remained low for the remaining of winter up into spring. The activities ranged from 1.5 to 3.0 U/g wet mass for HOAD, relatively similar to our values. In addition, the lipid content in *E. solidaginis* remained the same over the winter (Joanisse and Storey 1996). The same conclusions can logically be made in this study: *B. impatiens* queens do not fuel on lipid reserves during overwintering.

Changes in body composition

Bumblebee queens become lighter during the progression of overwintering, possibly due to the use of their energy reserves. However, the constant average dry mass of the abdomen indicates that the mass loss recorded in overwintering queens is mainly due to water loss. Since energy reserves are located in the fat bodies, the reduction in the abdomen mass should reflect the mobilization of fat or glycogen during the period where they are not feeding. In our study, the reduction of energy stores is not large enough to be detected by the abdomen mass loss. It has been documented that body mass is an important aspect of bumblebees' winter survival, and queens having larger mass also have a higher survival rate (Holm 1972; Vesterlund et *al*. 2014; Owen 1988). Owen (1988) showed that the body mass of eight different *Bombus* species was highest in the fall (before overwintering) and lowest in the spring (after overwintering). Following the idea that queens need to accumulate enough reserves in their fat bodies to survive all winter, queens that are heavier should have more success than smaller, lighter queens. Queens who do not have enough reserves (the lighter queens) are most likely to die before they can emerge in spring. The difference in wet mass between fall queens and spring queens was the first evidence of reserves being mobilized during overwintering for bumble queens (Owen 1988; Alford 1969) and presumably many other insects. Taking dry mass of queens at a random point in the overwintering process allows us to account for water loss; however, it may not be the most representative snapshot of a single individual going through winter. The fact is because heavier queens have a higher survival rate, the ones that are sampled further in the overwintering period are most likely queens that were heavier from the start. Sampled queens at the beginning of overwintering vary the most in terms of weight because they do not need to have many reserves at that point. Monitoring either the wet body mass or the dry abdomen mass of queens during

overwintering is not sufficient to conclude if there is, in fact, mobilization of energetic reserves during this period.

Surprisingly, we found no changes in any of the body composition measures used, namely glycogen, lipid, free carbohydrates, and protein concentrations. Previous studies have been successful in demonstrating a depletion of abdominal lipids in overwintering bumblebees (Alford 1969; Votavovà et *al*. 2015), other families of bees (Bosch et *al*. 2010; Weissel et *al*. 2012) and in wasps (Strohm 2000). Each of these studies had some differences with ours. Some studies sampled queens overwintering in their natural hibernacula and over a more extended period than typically measured in the laboratory (Alford 1969). The difference can also be in the approach to measure lipid content: lipids were extracted from fat bodies in chloroform and weighed (Votavovà et *al*. 2015), or it was calculated as the difference between the weigh before and after lipid extraction with petroleum ester (Strohm 2000; Weissel et *al*. 2012) or the fat bodies were X-rayed to score fat body conditions based on a semi-quantitative scale (Bosch et *al*. 2010). Based on these differences, a few hypotheses arise to explain the absence of energy stores depletion in our experiment. First, our laboratory settings for overwintering might be why we cannot see a decrease in metabolic reserves. In the study of Amsalem et *al*. (2015), overwintering bumblebee queen did not lose lipids or glycogen over a period of 9-10 weeks. They proposed the hypothesis that the artificial diapause regime might offer less physiological challenges on queens than in natural conditions. It has been demonstrated that temperature fluctuations affect lipid use in bumblebee queens (Vesterlund et *al*. 2014). Rises in temperatures are common throughout winter, and, as ectotherms, bumblebee's metabolism responds to ambient temperatures. When it gets warm, diapausing insects get more active and consume more of their energetic reserves

(Irwin and Lee 1999; Hahn and Denlinger 2007; Vesterlund et *al*. 2014). The temperature experienced by our queens in laboratory setting is relatively constant, the only fluctuations are coming from opening the incubator door once in a while to collect individuals The constant low temperature in experimental conditions will avoid semi-active bouts where the individual will use more metabolic fuels to survive (Hahn and Denlinger 2007; Hahn and Denlinger 2011). This could be a disadvantage of rearing queens in the laboratory. Another similarity in the study of Amsalem et *al*. (2015) with ours, is the method used to estimate lipids in the abdomen of queens. The vanillin-phosphoric acid reaction is commonly used but may not be the most appropriate method to quantify lipid concentrations in overwintering specimens (Williams et *al*. 2011). In the vanillin-phosphoric acid assay, triglycerides are broken down to fatty acids by sulfuric acid. The double bonds or hydroxyl groups on the fatty acids react with the sulfuric acid to form a chromogen. The chromogen then reacts with the vanillin reagent to form a chromophore that has an absorbance at 530 nm and can be compared to a standard curve (Johnson et al., 1977; Williams et *al*. 2011). This assay is best used to compare individuals because it will most likely under-estimate lipid reserves. The issue comes from polyunsaturated fatty acids that have a decreased reactivity, the more unsaturated they are, and saturated fatty acids that do not react with sulfuric acids and do not form a chromogen so cannot be detected (Williams et *al*. 2011). The plasticity of fatty acid profiles in response to the cold treatment may have introduced systematic biases. In *B. terrestris*, there was a decreased proportion of saturated fatty acids, while the proportion of monounsaturated fatty acid increased during its overwintering phase (Votavová et *al*. 2015). In freeze-avoiding moth, *E. scudderiana*, and freeze-tolerant, *E. solidaginis*, the degree of fatty acid unsaturation increased from September to December, as reflected by a double-bond index (Joanisse and Storey 1996). In all of these cases, estimating lipid reserves

with the vanillin-phosphoric acid assay would bias the results given the changes in numbers of double-bond. An actual decrease in lipid content could be masked by an increase in lipid saturation. There is a strong possibility that this was the case in our study. Another approach for quantifying lipid concentration during overwintering would be preferable in the future.

The capacity for carbohydrate oxidation remained the same throughout overwintering in bumblebee queens. The constant activity of HK, an enzyme involved in the first step of glycolysis, goes in pair with the respirometry assay, exhibiting pyruvate as a preferred substrate. HK activities, ranging around 50 U g^{-1} thorax, are similar to previous values reported by Billardon and Darveau (2019) and seem to be conserved throughout the entire life cycle of *B. impatiens* queens. In contrast, for freeze-tolerant larvae of *E. solidaginis*, there is evidence of metabolic reorganization taking place upon entry into diapause. HK activities decreased approximately fivefold from September to November and, from that point, increased steadily into the spring (Joanisse and Storey 1994). Changes in HK activity reflect the dynamic nature of the metabolism even in the dormant larvae at low ambient temperatures. *B. impatiens* queens maintain their capacity for glycolysis, but it does not translate into the use of carbohydrates during overwintering. There is no significant decrease in glycogen or free carbohydrates observed within four months of overwintering. Insect diapause is associated with qualitative and quantitative shifts in glycogen metabolism. Glycogen plays two major roles: it is converted into glucose or trehalose for transport out of the fat body to tissue for fueling catabolism, and it is mobilized to produce a variety of sugar-based cryoprotectant (Hahn and Denlinger 2011). Glycogen reserves were significantly decreased during cold winter months but not significantly different at the onset and termination of diapause for *O. bicornis* (Wasielewski et al. 2013). For

this mason bee species, glycogen is not used for fuel and is most likely transformed into cryoprotectants and recycled back to glycogen when temperature rises. In *B. terrestris*, glycogen reserves were also not significantly different at the onset and termination of diapause but were not measured during the diapause period (Amsalem et *al*. 2015). For *E. scudderiana*, cold exposure in the fall readily stimulated the production of glycerol, while glycogen reserves diminished. The cryoprotectant synthesis proceeded until glycogen concentration fell to low levels (Storey et al. 1990). In contrast, for diapausing individuals of two cool-temperate *Drosophila* species (*D. subauraria* and *D. triauraria*), glycogen remained rather high even in mid-winter but declined to low levels by spring. By contrast, the warm-temperate species (*D. rufa* and *D. Zutescens*) and the non-diapause strain of *D. triuraria* exposed to a cool-temperate climate lost more than half of their stored glycogen by mid-winter, and they did not survive to the spring (Kimura et al. 1992). It was concluded that glycogen levels play a part in winter survival in these *Drosophila* species. Our results do not demonstrate the conversion of glycogen into cryoprotectant molecules and also does not support the use of glycogen or free carbohydrates as metabolic fuel despite their high potential for carbohydrate oxidation.

Conclusion

In this study, we investigated the role of proline as a metabolic fuel for overwintering B. impatiens. Multiple factors brought us to this question. First, there was some discrepancy regarding the use of lipids during bumblebee queens' overwintering despite their apparent inability to oxidize it. Then proline came as a viable hypothesis given it is a known fuel for some insects, and it can be resynthesized from lipids. While demonstrating that bumblebee muscle mitochondria cannot oxidize lipids, we could not show that proline was an important fuel for overwintering queens. However, more tests should be done, especially respirometry assays at lower temperatures, before the proline hypothesis is completely ruled out as a key feature of the bumblebee overwintering metabolism.

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