The role of proline oxidation and metabolome dynamics during the flight of *Bombus impatiens*

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AAT – alanine aminotransferase

G6P – glucose-6-phosphate

F6P – fructose-6-phosphate

FBP – fructose-1,6-bisphosphate

DHAP – dihydroxyacetone phosphate

G3P – glyceraldehyde-3-phosphate

3BPG – 1,3-bisphosphoglycerate

3PG – 3-phosphoglycerate

2PG – 2-phosphoglycerate

PEP – phosphoenol pyruvate

NADH/NAD – nicotinamide adenine dinucleotide

ATP – adenosine triphosphate

ADP – adenosine diphosphate

AMP – adenosine monophosphate

cGMP – cyclic guanosine monophosphate

MeOH – methanol

ACN – acetonitrile

H2O – water

FA – formic acid

EtOH – ethanol

Da – Daltons

LC-MS – liquid chromatography-mass spectrometry

UPLC – ultra performance liquid chromatography

MS – mass spectrometry

Q-TOF – quantitative time-of-flight

ESI (+/-) – electrospray ionization (positive or negative mode)

PPM – particles per million

DI – distilled water

OD – optical density

PCA – principal component analysis

PC – principal component

Abstract

Several insect species can use the amino acid proline as a major energy substrate, a unique characteristic of these animals. Although initially thought to be limited to blood feeding dipterans, studies revealed this capability may be more widespread. Recent work showed that the bumblebee *Bombus impatiens* can oxidize proline at a high rate, as measured using isolated flight muscle. However, its role as a metabolic fuel to power flight is unclear. To elucidate the extent to which proline is oxidized to power flight and how its contribution changes during flight, metabolites of central carbon and proline metabolism were profiled at key time points in hemolymph and flight muscle tissue. Analysis using UPLC-MS-QTOF has revealed trends in fuel use and changes in pathway metabolites. Of 29 targeted metabolites, 18 were detected in flight muscle tissue. Two flight experiments were conducted and concentrations of metabolites at the end of prolonged flight are similar to those at rest, or have decreased significantly. In total, 14 of 19 metabolites significantly changed in concentration. The results correspond to a model of fuel use during flight, which states that proline is oxidized at the onset of flight, then carbohydrates take over as the main fuel, accompanied by a decrease in glycogen. By 8 minutes of flight, metabolite concentrations stabilize and flight performance does not change. Patterns in metabolite fluctuations suggest proline is used to supplement the Krebs cycle, and carbohydrates are the main fuel, maintained by glycogen stores. This indicates homeostatic regulation of intermediates and replenishment of fuels, or depletion of fuels due to their recruitment for ATP generation. This targeted metabolomics approach will clarify the role of proline and carbohydrate metabolism and pathway regulation during flight in *B. impatiens*.

Résumé

Plusieurs espèces d'insectes peuvent utiliser l'acide aminé proline comme substrat énergétique majeur, une caractéristique unique de ces animaux. Bien que l'on pensait au départ que cette propriété se limitait aux diptères hématophages, des études ont révélé que cette capacité pourrait être plus répandue. Des travaux récents ont montré que le bourdon *Bombus impatiens* peut oxyder la proline à un taux élevé, tel que mesuré en utilisant le muscle du vol isolé. Cependant, son rôle en tant que carburant métabolique pour le vol n'est pas clair. Pour déterminer dans quelle mesure la proline est oxydée pour le vol et comment sa contribution change en vol, les métabolites du carbone central et du métabolisme de la proline ont été profilés, à des moments clés lors du vol, dans l'hémolymphe et le tissu musculaire associé au vol. L'analyse utilisant UPLC-MS-QTOF a révélé des tendances dans l'utilisation du carburant et des changements dans les métabolites des voies. Des 29 métabolites ciblés, 18 ont été détectés dans le tissu musculaire de vol. Deux expériences de vol ont été menées et les concentrations de métabolites à la fin d'un vol prolongé sont similaires à celles observées au repos, ou ont diminué de manière significative. Au total, 14 des 19 métabolites ont significativement changé de concentration. Les résultats correspondent à un modèle d'utilisation du carburant en vol, qui indique que la proline est oxydée au début du vol, puis les hydrates de carbone prennent le relais comme carburant principal, accompagné d'une diminution du glycogène. En 8 minutes de vol, les concentrations de métabolites se stabilisent et les performances de vol ne changent pas. Les modèles de fluctuations des métabolites suggèrent que la proline est utilisée pour compléter le cycle de Krebs, et que les glucides sont le combustible principal, maintenu par les réserves de glycogène. Ceci indique la régulation homéostatique des intermédiaires et la reconstitution des carburants, ou l'épuisement des carburants en raison de leur recrutement pour la production d'ATP. Cette approche métabolomique ciblée clarifiera le rôle du métabolisme des prolines et des glucides ainsi que la régulation des voies métaboliques durant le vol chez *B. impatiens*.

Introduction

Overview

Animals are capable of using a variety of energy substrates to fuel cellular metabolism, and studies in insect biochemistry have documented numerous ways in which insect flight is fueled. Carbohydrates, lipids, and even proteins have been cited as the predominantly used fuels for this intensive aerobic activity, in bees, locusts, and blood-feeding insects, respectively. Many species are also capable of metabolizing amino acids like proline to fuel their activities. Across the order Hymenoptera, proline metabolism is not well documented. The honeybee *Apis mellifera* is often used to exemplify this order. They are almost exclusively carbohydrate users, and proline has demonstrated minimal contribution to total energy use during flight (Barker & Lehner 1970). Recent work in isolated insect flight muscle tissues has revealed that common eastern bumblebees (*Bombus impatiens*) and wasps (*Vespula vulgaris*) are two hymenopterans capable of oxidizing proline to a large extent (Teulier et al., 2016). It remains to be elucidated if bumblebees are capable of using proline to fuel flight *in vivo,* and whether the high energy demands of flight has resulted in proline use as a metabolic adaptation. The goal of this study is to clarify the role of proline in bumblebee flight. Changes in proline and carbohydrate metabolic pathways will be profiled at various stages of flight to understand their capacity to fuel flight.

Proline metabolism in insects

Insects are capable of using a variety of fuels for flight, ranging from carbohydrates, proteins, and lipids. Often, these fuels are coupled with each other based on the requirements of aerobic flight muscle metabolism, and the pairings allow for maximal functioning of the pathways

that produce energy (Storey 1985). For example, locusts fly for extended periods of time, and although they initially start by using carbohydrate as the main fuel, eventually they make a switch to lipids due to its high energy content (Van der Horst et al., 1980). Some insects are also capable of using amino acids as a source of energy. In particular, proline is the amino acid of choice. It is found in large quantities in insect tissues (Micheu et al., 2000) despite variations in diet, whether the insect is nectar- or blood-feeding. Although for most insects the role of proline metabolism is still unknown, there are several options for the use of proline as an energy substrate. Proline can be used exclusively or as the major fuel, as in the case of the blood-feeding tsetse fly, *Glossina morsitans*, since blood is rich in proteins and amino acids (Auerswald and Gäde, 1999). Their mitochondria are capable of oxidizing proline about 100 times as fast as pyruvate (Kammer and Heinrich, 1978). In the case of species that do not feed on blood, or if only the female consumes blood, proline is oxidized at a slower rate than other substrates (Bursell, 1975). For example, in mosquitos (*Aedes aegytpti*), only the female consumes blood, and male mitochondria demonstrate a 38% lower respiration rate than female in the presence of proline with pyruvate (Soares et al., 2015). However, in the females, pyruvate is oxidized at a much faster rate than proline, so although proline can be used for flight, feeding on proline-rich blood is likely for reproductive purposes (Bursell, 1975). There are exceptions to this scheme, though. Proline can also be used as a "sparker" to the Krebs cycle, whereby proline augments Krebs cycle intermediates required for acetyl-CoA oxidation (Storey 1985, Auerswald and Gäde, 1999). In this case, it is often used in conjunction with another fuel, such as carbohydrates. For example, the blowfly *Phormia regina* uses proline as a sparker to supplement the Krebs cycle intermediates, particularly at the start of flight. Evidence for this function has been collected by Johnson and Hansford (1975) by supplying proline and pyruvate to blowfly mitochondria, which resulted in an increase in the total content of

Krebs cycle intermediates. In the Colorado potato beetle (Weeda et al., 1979) and African fruit beetle (Zebe and Gäde, 1993), proline can be used simultaneously with carbohydrates.

Proline oxidation occurs by catalysis through proline dehydrogenase, which ultimately leads to glutamate production. Partial proline oxidation is unique in the sense that the glutamate acts as a substrate for alanine aminotransferase (AAT), which creates the α-ketoglutarate that is required for acetyl-CoA oxidation through the Krebs cycle (Zebe and Gäde, 1993). Pyruvate is generated from malate through the malic enzyme, and is subsequently converted into alanine by AAT. This alanine is resynthesized into proline, the original fuel, making it an osmotically neutral reaction. The high solubility of proline and concentration of proline in both flight muscle and hemolymph make it readily available as a fuel, and it does not require specific carrier proteins either (Weber 2011; Gäde and Auerswald, 2002). Proline can also act as a carbon shuttling molecule between lipid stores in the fat body and muscles (Gäde and Auerswald, 2002; Bursell 1977). Lastly, partial proline oxidation does not create nitrogen waste products. The ways in which proline appears to be oxidized in most insects is a partial oxidation (as a sparker, exclusive proline use, or in combination with carbohydrates). However, proline can also undergo complete oxidation. This process requires the activity of the enzyme glutamate dehydrogenase and creates ammonia as a result (Storey 1985). Evidently, proline can be more than just a dietary opportunity for insects. It is effective as a fuel due to its high energy content – the partial oxidation of proline creates 0.52 mol of ATP/g, which makes it more similar to lipids (0.65 mol/g) than carbohydrates (0.18 mol/g) (Gäde and Auerswald, 2002).

Proline as an energy substrate in bees

The honeybee *A. mellifera* is often used to exemplify other Hymenoptera. It is well known that honeybees rely almost exclusively on carbohydrates to fuel their activity, since plant nectars are rich in sugars. The use of proline as a metabolic fuel has also been documented, though it has been found that proline does not largely contribute to total energy use. Barker and Lehner (1972) estimate that only 0.1% of flight energy of worker honeybees is generated through proline. Micheu et al. (2000) report findings that also suggest honeybees may use proline. Although the concentration of proline in flown bees was significantly lower than in rested bees, the amount metabolized was much lower compared to the use of carbohydrates. Crailsheim and Leonhard (1997) suggest that in foragers, the decrease in proline seen after returning from flights may be indicative of proline use in foraging metabolism. Furthermore, its possible that honeybees are not using proline as a fuel to a larger extent because their flight muscles do not contain sufficient proline dehydrogenase (Crabtree and Newsholme, 1970). Evidently, carbohydrates are the fuel of choice because glycogen and carbohydrates can be metabolized in muscles immediately to maintain ATP levels (Kammer and Heinrich, 1978).

Plant physiologists have noted that the nectars of pollinator-attracting plants are not only rich in sugars, but are also rich in proline relative to other amino acids – it occurs at concentrations of 2mM (Carter et al., 2006). Based on this finding, it is possible that proline-rich nectars act as a metabolic reward, a mechanism to attract pollinators like bees. The muscle metabolism of other bee species has not been as widely explored as that of the honeybee, so the idea that Hymenoptera such as the bumblebee would use fuels other than carbohydrates seemed unlikely. A recent study by Teulier et al. (2016) observed the ability of several hymenopterans to oxidize proline *in vitro*. Using isolated flight muscle tissues, it was clear that bumblebees (*Bombus impatiens*)

demonstrated a large potential for ATP production through proline oxidation. Respiration rates more than doubled when proline was added to the mixture of substrates responsible for cellular respiration. This finding supports the hypothesis that hymenopteran pollinators might use proline in nectars as a metabolic reward. However, the proportions with which insects can use proline varies widely among species (Storey 1985) and the capacity for proline metabolism may be similarly diverse among bee species. Even within the same species, there are differences in proline concentration preferences between colonies (Carter el al., 2006), so although this amino acid is widespread in insect nutrition choice, it may be sought out and used to different extents. It remains to be elucidated if *B. impatiens* uses proline as an energy substrate for flight, or if the phenotype for proline oxidation evolved for other purposes in this species. The capacity to use proline as a way to enhance the oxidation of carbohydrates has been documented previously in both dipterans (van den Bergh 1964; Brosemer and Veerabhadrappa 1965; Sacktor and Childress 1967, Scaraffia and Wells, 2003; Soares et al., 2015) and coleopterans (Weeda et al., 1980; Weeda 1981; Auerswald and Gäde 1999; Gäde and Auerswald 2002), so there is a possibility that bumblebees use proline as a fuel in combination with carbohydrates that they feed on prolifically. Furthermore, the closely related wasp *Vespula vulgaris* oxidized proline to a similar extent as bumblebees, suggesting that this phenotype is more widespread. Honeybees may not be the best model to exemplify energy metabolism in Hymenoptera. The goal of this study is to better understand the role of proline oxidation during flight, and observe the changes in the concentration of intermediates and metabolites of various pathways involved in sustaining strenuous activity.

Targeted metabolomics as a profiling technique

In studies done by Sacktor and Wormser-Shavit (1966) and Sacktor and Hurlbut (1966), the flight of blowflies was profiled for an hour. Through spectrophotometric measurements, glycolytic, Krebs cycle, amino acid intermediates, adenine nucleotides, arginine phosphate, and inorganic phosphate were recorded as they changed throughout the duration of flight. These experiments shed light on how all these metabolites and intermediates are involved in the flight of *P. regina*, and highlight the "sparker" role of proline early in this dipteran's flight. By observing the changes in metabolites of several metabolic pathways, it is possible to gain insight into the overall dynamic of the metabolome during a physiological demand such as flight. The metabolome includes all the chemicals in a biological system with molecular masses below 1500 Da, and they can be detected through techniques like mass spectrometry (Barnes et al., 2016). Essentially, these are intermediates of metabolic pathways, rather than the large storage molecules from which they may be derived (i.e. glycogen, triglycerides). The study and global measurement of all the metabolites is metabolomics, a newer omics technology. However, the approach can be more targeted. Essentially, it is a way to summarize larger changes in cell phenotypes by observing changes in a smaller number of metabolic pathways (Snart et al., 2015). Metabolomics is being employed more often in the context of entomology, from behavioural studies, insect-fungus interactions, temperature stress responses, and more. For example, Xu et al. (2015) found that fungal propagation in silkworm larvae significantly alters energy metabolism. Metabolomic data revealed upregulation of energy metabolites like carbohydrates, amino acids, and lipids, while downregulating eicosanoids and amines, suggesting that the fungus causes nutrient deprivation and suppresses host immune response. The use of metabolomics can offer novel insight into biological processes involving smaller molecules (Nicholson and Wilson, 2003).

In the case of the bumblebee, in order to elucidate the role of proline in flight metabolism, the metabolites and intermediates of several pathways must be taken into consideration. These include the proline catabolic pathway, the glycolytic pathway, the Krebs cycle, and intermediates involved in oxidative phosphorylation – similar to what was tracked in *P. regina*. Metabolomics generally has some advantages over more established omics techniques. Due to its focus on downstream cellular functions, it is possible to observe the functional metabolic phenotype of organisms without requiring prior knowledge of the genome (Snart et al., 2015). With techniques like liquid chromatography-MS, it is possible to adjust solvent systems in order to detect as many metabolites of interest as possible. As a result, it is possible to collect information about biochemical pathways and fluctuations in their intermediates without doing full characterisations (Snart et al., 2015). By generating profiles of the metabolome during flight at time points that are considered key metabolic transition periods, it is possible to create snapshots of the functional metabolic phenotype during prolonged flight, on a more concise, targeted scale. Through this process, it may be possible to identify the relationships between phenotypic states and the cellular metabolism of bumblebees, and ultimately gain a better understanding of the role of proline metabolism in *B. impatiens*.

Questions and hypotheses

Metabolic profiles of bumblebees at various stages of flight were generated using UPLC-MS-QTOF. The goal of the project was to answer the questions: to what extent is proline oxidized to power flight, and how does the role of proline change throughout the duration of flight? In order to answer these questions, metabolic profiles of bees at various stages of flight were generated

using UPLC-MS-QTOF. We hoped to observe the fluctuations in metabolite intensities *in vivo*, that might complement the *in vitro* findings of Teulier et al. (2016). The hypotheses for these experiments are that during the initiation of flight, flight muscle proline concentration will decrease and simultaneously, flight muscle glucose concentration will also decrease. In addition, metabolites are constantly in flux despite lack of noticeable change over time. If an external stimulus is large or sudden enough (i.e. onset of flight), we expect that there will be a significant change in metabolite concentration. Thus, we hypothesize that if a biochemical pathway is recruited during flight, the concentration of its respective metabolites will change significantly.

We predicted that like in other insects, proline will be recruited during flight, particularly during the early stages of flight. Furthermore, metabolites of the carbohydrate and Krebs pathways will change due to their involvement in ATP generation during activity. Zebe and Gäde (1993) propose a model for flight energy metabolism in coleopteran flight muscles and abdomens, including three distinct phases. During the first few minutes of flight, proline is used as the main substrate and alanine accumulates as an end product. Next, there is a large decrease in glycogen content due to the increased demands of carbohydrate metabolism. Lastly, metabolite levels begin to stabilize at about 8 minutes of flight, without noticeable change in flight performance. We expected that carbohydrates in the bee will follow this pattern with a sharp decrease seen early in flight, since it is expected they use proline only in addition to carbohydrates.

Methods

Insects and holding conditions

Bombus impatiens colonies (Biobest, Leamington, ON, Canada) containing one queen and 20 workers were maintained at 22°C in plastic hives stored in cardboard boxes with lids. Bees were fed a diet of pollen (pollen ground with a mortar and pestle, mixed with sugar water, molded into small spheres) and reservoir provided by Biobest. Bees were allowed to feed ad libitum. Adult worker bees (females) were used for all experiments.

Flight experiments

Bees were immobilized by cooling in a 4°C fridge in a 50ml conical tube for 30-40 minutes. A needle was fixed to the top of the thorax, between the wings, using UV-cured resin (Solarez, Vista, CA, USA). Bees were tethered to a flight mill and allowed to adjust to room temperature of 22°C, at which flights were conducted.

Flight was stimulated by the tarsal and optomotor reflexes. The tarsal reflex occurs when the tarsi are detached from a surface, mimicking free-fall and triggering flight (Meresman et al., 2014). The optomotor reflex is used for stabilization during free locomotion to regain the desired course of movement (Lehrer 1993), which was stimulated by a visual panorama. However, if flight was irregular or discontinuous, the individual was returned to the colony. The initially selected duration of continuous flights was 2, 8, 15, and 30 minutes, and a control group of resting bees (0 min). Results obtained from this first experiment will thereafter be referred to as the first flight experiment. A second flight experiment was performed with the same flight durations as above, a

control rest group, plus flight times of 5-10 seconds and 30 sec. Individuals reached the desired flight time at continuous or near-continuous flight (flight was re-initiated within 30s-60s). They were immediately clamped with tongs that had been chilled in liquid nitrogen, and then dipped in liquid nitrogen to thoroughly freeze the tissues. These frozen bees were then stored at -80°C.

To collect hemolymph, bees were immediately placed into a 50ml tube at the end of flight, which was then injected with nitrogen gas in order to immobilize the bees. The abdomen was gently cut between the 3rd and 4th abdominal segments with dissection scissors, and 4-5µL of hemolymph was drawn out with a pipette. Hemolymph was transferred into individual microcentrifuge tubes and frozen in liquid nitrogen, then stored at -80°C.

Preparation of analytical standards

In preliminary experiments, analytical standards (Sigma-Aldrich, Oakville, ON, Canada) were prepared for a total of 29 targeted compounds that spanned the categories in Table 1. Each standard was prepared as a stock at a concentration of 1 mg/mL by dissolving it in a solvent system through vortexing or sonification. The final solvent system chosen was a 40:40:20 mixture of methanol, acetonitrile, and water (Fisher Optima LC-MS, Brockville, ON, Canada). Preliminary experiments tested a second system (40:40:20 of methanol, acetonitrile, water+0.1% formic acid), and the former was chosen due to greater extraction of metabolites. Subsequently, standards were diluted to 0.01, 0.1, 1, 10, and 100 µg/mL. All concentrations of standards were stored in glass vials at -80°C.

Table 1. Analytical standards prepared for targeted metabolites.

Pathway	Metabolite
Amino acids	proline, alanine, glutamate
Carbohydrates	glucose, fructose, trehalose
Glycolytic intermediates	glucose-6-phosphate (G6P), fructose-6-phosphate (F6P), fructose-1,6-bisphophate (FBP), dihydroxyacetone phosphate (DHAP), glyceraldehyde-3-phosphate (G3P), 1,3-bisphosphoglycerate (3BPG), 3-phosphoglycerate (3PG), 2-phosphoglycerate (2PG), phosphoenol pyruvate (PEP), pyruvate
Oxidative phosphorylation	nicotinamide adenine dinucleotide (NADH), adenosine triphosphate (ATP), adenosine diphosphate (ADP), adenosine monophosphate (AMP), cyclic guanosine monophosphate (cGMP)
Krebs cycle intermediates	acetyl-coA, citrate, α-ketoglutarate, succinyl-coA, succinate, fumarate, malate, oxaloacetate

Preparation of samples

Frozen bees were quickly dissected, and the thoraces and abdomens were isolated. Each component was weighed and placed in an individual centrifuge tube and filled with 9 volumes of solvent mixture. The thoraxes and abdomens were minced with scissors and homogenized (Polytron PT 1300 D). It was then sonicated for 3 minutes using 5 second pulses at 30 sec intervals (Sonics Vibra-cell). The homogenate was centrifuged for 15 minutes at 10 000 x g and 4°C. The supernatant was collected and transferred to glass vials on ice. To maximize the extraction of metabolites, another 9 volumes of solvent were added to the pellet. The pellet was sonicated and centrifuged in the same manner. The supernatant was collected and combined with the supernatant from the first extraction to create a 1:20 dilution. This was done in order to extract as many metabolites as possible from the muscle tissue and abdomens. The supernatant was stored at -80°C in glass vials.

Hemolymph was thawed on ice and processed similarly: 4 µL was added to the same solvent systems to make 1:20 dilutions, and sonicated in a water bath for 3 minutes. It was centrifuged for 15 minutes at 10 000 x g and 4°C. The supernatant was collected and stored on ice in glass vials. Solvent was added to the hemolymph pellet for a second extraction, and the resulting supernatant was combined with that from the first collection. Samples were stored at -80°C in glass vials. Hemolymph samples were collected only in the first flight experiment, at the same flight times as tissues. Abdomen samples were only included in the second flight times, at time points 0 and 30 min.

All concentrations of standards were plated into a 96 well plate (Waters Inc., Milford, MT, USA). Samples were centrifuged once again prior to plating: 300 µL of supernatant of each sample was transferred to individual centrifuge tubes and centrifuged for 15 minutes at 10 000 x g and 4°C. Then, 200 µL of the supernatant was pipetted into a syringe fitted with a syringe-driven filter unit (Millex, 0.45 um, 4 mm diameter, PTFE membrane, Millipore, Billerica, MA, USA). The supernatant was then loaded into the 96 well plate. All plates were sealed and stored at -80°C until injection.

Ultra-performance Liquid Chromatography-quadrupole time-of-flight mass spectrometry analysis

Ultra-Performance Liquid Chromatography-quadrupole time-of-flight mass spectrometry (UPLC-Q-TOF) analyses were undertaken on an Acquity UPLC coupled with XevoG2 QTOF system (Waters Inc., Milford, MT, USA). Separations were performed on a BEH C18, 1.7 µm particle size, 2.1 ×100 mm column connected with a VanGuard pre-column, 2.1 x 5 mm.

Mobile phase A (water + 0.1% formic acid) and B (acetonitrile + 0.1% formic acid) (Fisher Optima LC-MS, Brockville, ON, Canada) were delivered at a flow rate of 0.8 mL/min at a column temperature of 65°C, with the sample temperature at 4°C. Mobile phase A was delivered isocratic 100% 0-3 min, linear gradient 0-20% B 3-5 min, 100% B isocratic 5-6 min. A 5 µL injection was performed through a 10 µL loop followed by a strong wash of 200 µL (50% acetonitrile + 50% water) and weak wash of 600 µL (10% acetonitrile + 90% water).

Q-TOF was operated in positive and negative electrospray ionization (ESI) modes. MassLynx software (Version 4.1) was used to acquire high and low energy spectra in MS^e ESI+ and MS^e ESI- modes within the mass range of 100-1000 Da. Cone voltages were 15V in both positive and negative modes, while scan time was set at 0.08 seconds.

Lock mass was set with Leucine Enkephalin C^{12} at 556.2615 Da $[M+H]^{+1}$ and 554.261 Da $[M-H]^{-1}$. Source and desolvation temperatures were 150°C and 500°C, respectively. Cone gas and desolvation gas (nitrogen) were set at 50 and 1200 L/hr. The molecular ions were acquired at low fragmentation (6V) and the product ions at high fragmentation (20-40V). A mass accuracy threshold of 5 PPM and an ion intensity threshold of 1000 was used as criteria for the identification and detection of target compounds, respectively.

Detection of analytical standards

Of the 29 targeted metabolites, 18 were detected. The standards that were successfully detected using thresholds set for UPLC-MS analysis are listed in Table 2.

Table 2: Detected analytical standards.

Pathway	Metabolites
Amino acids	proline, alanine, glutamate
Carbohydrates	glucose, fructose, trehalose
Glycolytic intermediates	G6P, F6P, DHAP, G3P, 3PG, pyruvate
Oxidative phosphorylation	AMP, cGMP
Krebs cycle intermediates	α-ketoglutarate, succinate, fumarate, malate

Determination of glycogen content in abdomens

To determine the glycogen content of single bumblebee abdomens, a protocol adapted from Van Handel (1985), Kaufmann and Brown (2008), Lorenz (2003), and Panzenbock and Crailsheim (1997) was followed. Standard curves were generated from anhydrous glucose (100 mg per 100 mL distilled water [DI]). The glucose standard curve ranged from 0.005 mg/mL to 0.04 mg/mL. Anthrone reagent (385 mL 98% sulfuric acid added to 150 mL DI, 750 mg anthrone mixed in) was added to the 2-mL mark and vortexed briefly.

For tissue samples, the abdomens were separated from the rest of the frozen body, weighed, and individually homogenized in a centrifuge tube in a mixture of 200 µL 2% sodium sulfate solution (2 g in 98 mL DI), 200 µL 70% EtOH, and 300 µL 80% MeOH. Abdomens were minced with scissors and homogenized on ice until no identifiable parts remained. Homogenate was sonicated in 5 second pulses 6 times, with 30 second intervals between pulses. The homogenate was then heated for 30 min at 70°C to completely solubilize any remaining glucose and deactivate enzymes, vortexed every 10 min. After cooling at 4°C for 30 min, the mixture was centrifuged for 10 min at 4°C and 21 000 x g. The supernatant was discarded, and free glucose was rinsed off

homogenate with 80% methanol (2 x 400 µL). 200 µL sodium sulfite solution and 300 µL EtOH was added and the sample was vortexed, sonicated, then vortexed again. The mixture was centrifuged, and the supernatant discarded. The pellet was completely dried on the thermoblock at 80°C, and then 600 µL DI was added. After vortexing, the homogenate was left standing for 10 min to allow glycogen to dissolve.

The homogenate was vortexed again prior to subsampling and 120 µL of the fluid was transferred to a clean centrifuge tube, and 480 µL of anthrone reagent was added. The mixture was vortexed. Both samples and standards were heated for 17 minutes at 99°C, and after removal from the heating block and cooling, the optical density was measured at 625nm (BioTek Synergy 2 spectrophotometer).

Data collection and statistical analysis

Data collection from the UPLC was performed with MassLynx software (Waters, version 4.1). The relative ion abundance (intensity) of each targeted metabolite was determined from the chromatograph of each analytical standard and tissue, abdomen, or hemolymph sample. Metabolite intensity was also collected from the analytical standards that were injected in order to create standard curves. Statistical analysis was performed using Systat 12. Analysis of variance (ANOVA) was conducted, followed by post-hoc tests using Tukey's Honestly-Significant-Difference test in order to determine differences in metabolite intensities among the different flight times. Levene's test was used to check for homogeneity among variances, and if there were unequal variances, the Games-Howell test was used for pair-wise comparison. Linear regressions were used to generate standard curves and in turn, calculate metabolite concentrations. Some

results were reported as signal intensities rather than as concentrations. This is for one of two reasons: either the metabolite was entirely not detectable within the range of the standard curve, or many individuals did not fall within this range and excluding them from statistical analysis would skew results and statistical analysis

A portion of data analysis was completed using MetaboAnalyst 3.0 (http://www.metaboanalyst.ca), a web-based tool for the processing and analysis of metabolomic data (Xia & Wishart, 2011). Using this program, the data was normalized (normalization by sum) and scaled (autoscaling for column-wise normalization) in order to reduce systematic variance and conduct multivariate analyses. Furthermore, this normalization transforms peak intensity values so their distribution is more Gaussian or normal, and they share a mean, standard deviation, and upper range (Xia and Wishart, 2011). A Principle Component Analysis (PCA) was completed with the metabolite datasets for thoraxes, reducing all the variables to the first two principal components. Biplots were generated first, with vector labels corresponding to the mass or mass+adduct of the metabolite. The correlation of variables is determined by angles between the vectors, so the closer the points are, the more correlated. The magnitude of the effect is demonstrated by the length of the vector – a longer vector suggests the effect of the independent variable was greater. Scores plots were created to visualize time-based clustering and shifts in metabolic profiles throughout the duration of flight. Each point on the scores plot represents one individual's metabolic profile.

Results

Overview of detected metabolites in sample types

Of the total 18 detected compounds in the standards library, the largest number of metabolites detected confidently was the same 18 metabolites, in the thoracic tissue. All detected metabolites are presented in Table 3 by sample type. Although the metabolites AMP, DHAP, G3P, and 3PG were detected in the standards library, this did not occur in some sample types at a signal intensity with which it could be confidently reported as present. Important to note is that the tissue extractions are contaminated with hemolymph. Although metabolites in the hemolymph are contributing to overall metabolite signals, it is likely to a much lesser extent as those in thoracic muscles or other abdominal contents.

Table 3. Metabolites detected in each sample type.

Sample	Thorax	Abdomen	Hemolymph
Metabolites	proline	proline	proline
	alanine	alanine	alanine
	glutamate	glutamate	glutamate
	trehalose	trehalose	trehalose
	glucose	glucose	glucose
	fructose	fructose	fructose
	AMP	AMP	
	cGMP		cGMP
	G6P	G6P	G6P
	F6P	F6P	F6P
	pyruvate	pyruvate	
	succinate	succinate	succinate
	malate	malate	malate
	DHAP		
	G3P		
	3PG		3PG
	α-ketoglutarate	α-ketoglutarate	α-ketoglutarate
	fumarate	fumarate	fumarate
Total	18	14	14

Overall metabolite dynamics

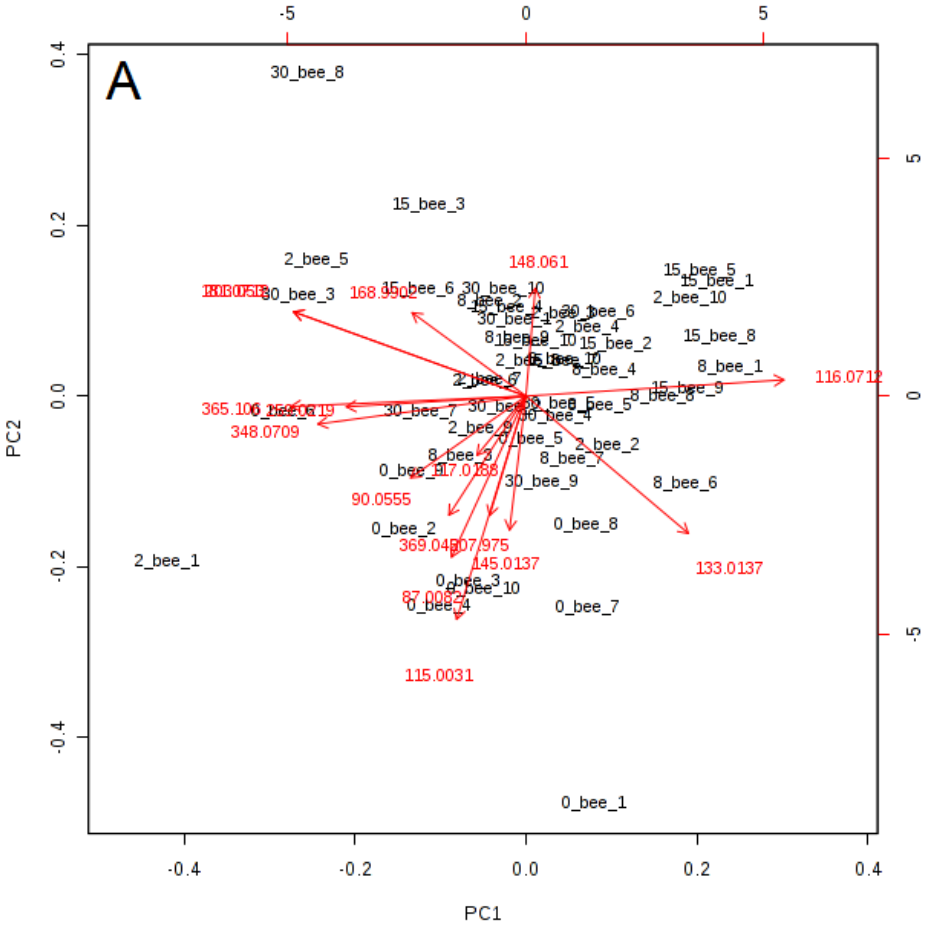
Principal component analyses were performed in order to determine overall metabolite changes in all the individuals over time. In the first flight experiment, 16 metabolites were reduced to the first two principal components that account for 48.6% of the variability. Based on the biplot (Fig. 1A), it appears that PC1 is dominated by trehalose (365.106), G6P and F6P (259.0219), AMP (348.0709), as well as proline (116.0712). Vectors that point in the same direction correspond to variables sharing similar response profiles. Trehalose and two glycolytic metabolites share a profile, which indicates that the changes observed in these metabolites are more likely to be correlated to the independent variable of flight time. The vector describing proline extends in the opposite direction from the other PC1-dominating metabolites, indicating that the changes seen in proline would be opposite to changes in the trehalose, G6P, and F6P.

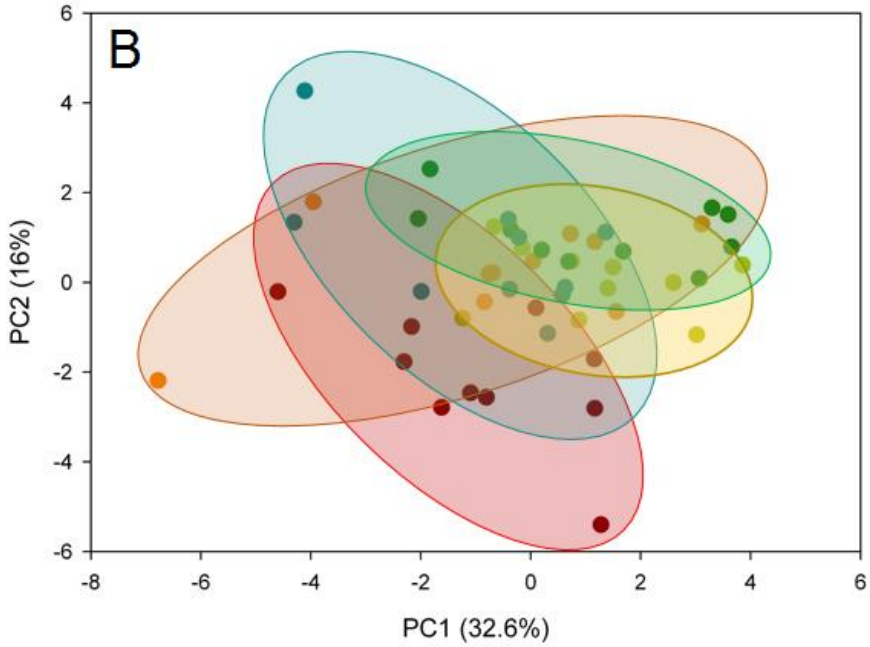
PC2 appears to be dominated by the metabolites succinate (115.0031), pyruvate (87.0082), α-ketoglutarate (145.0137), and cGMP (369.0450). The vector describing glutamate (148.061) extends in the opposite direction. The magnitude of their response to the effect of flight time is less than what is seen in PC1, due to short vector lengths. However, the behaviour of the vectors corresponds to the shallower decreases in these metabolites (which also appear to show an increase at the 8-min mark), and the fact that glutamate increases at the times where the other metabolites show decreases.

In the scores plot (Fig. 1B), individuals are represented by the points and are grouped by flight time. The shifts in the clusters at the different time points are indicative of the overall metabolite profile changes. For example, the cluster surrounding the bees flown for 2 minutes shows the largest variation, as it spans the entirety of PC1. Amongst individuals, the most variation

at metabolite profiles may be occurring at 2 minutes of flight. It is clear that a large change also occurs between rest and 2 minutes of flight, as the direction of the cluster changes so that they are almost perpendicular to each other. This may correspond to what is seen in the biplot with trehalose, G6P, F6P, and proline. These metabolites collectively contribute to most of the variation seen in the dataset, and may result in the variation seen at the 2 minute cluster. Within this cluster are also the 8 and 15 min cluster. These two clusters largely overlap, indicating less change occurs between these time points. Furthermore, they are much more compact than remaining time points, suggesting that there is less variation between individual profiles at these times. Collectively, they show good separation from the 0 min cluster, indicating that halfway through the flight experiment, enough change has occurred that there is a noticeable difference in metabolite quantities. The cluster representing 30 min does not show much separation from 0 min, indicating that there is not much variation between the two groups. This is likely due to the fact that many metabolites tend to either increase or decrease in quantity to similar amounts seen at rest.

Additionally, the changes in the mean scores of PC1 and PC2 were plotted over the course of time. In the PC1 scores, an ANOVA test revealed that although there is some significance to the results ($F_{4,45} = 2.591$, $p = 0.049$, Fig. 1C), pair-wise comparison showed no significant separation between metabolic profiles throughout flight ($p > 0.05$). However, in the PC2 scores, there is a significant increase ($F_{4,45} = 11.558$, $p < 0.001$, Fig. 1D) in the score from rest to all other flight times. This suggests that perhaps larger shifts occur in a different subset of metabolites in the overall profile at the onset of flight, since PC2 shows significant change despite accounting for less of the variation in data (16%).


A
PC1
PC2
30_bee_8
15_bee_3
2_bee_5
148.061
30_bee_3
168.9908
15_bee_6
30_bee_10
15_bee_5
15_bee_1
2_bee_10
15_bee_2
15_bee_8
8_bee_1
116.0712
8_bee_4
15_bee_9
8_bee_8
30_bee_7
2_bee_9
348.0709
8_bee_3
8_bee_7
2_bee_2
30_bee_9
8_bee_6
90.0555
0_bee_2
0_bee_8
369.0407.975
145.0137
133.0137
2_bee_1
0_bee_3
0_bee_10
0_bee_4
0_bee_7
115.0031
0_bee_1


B
PC1 (32.6%)
PC2 (16%)

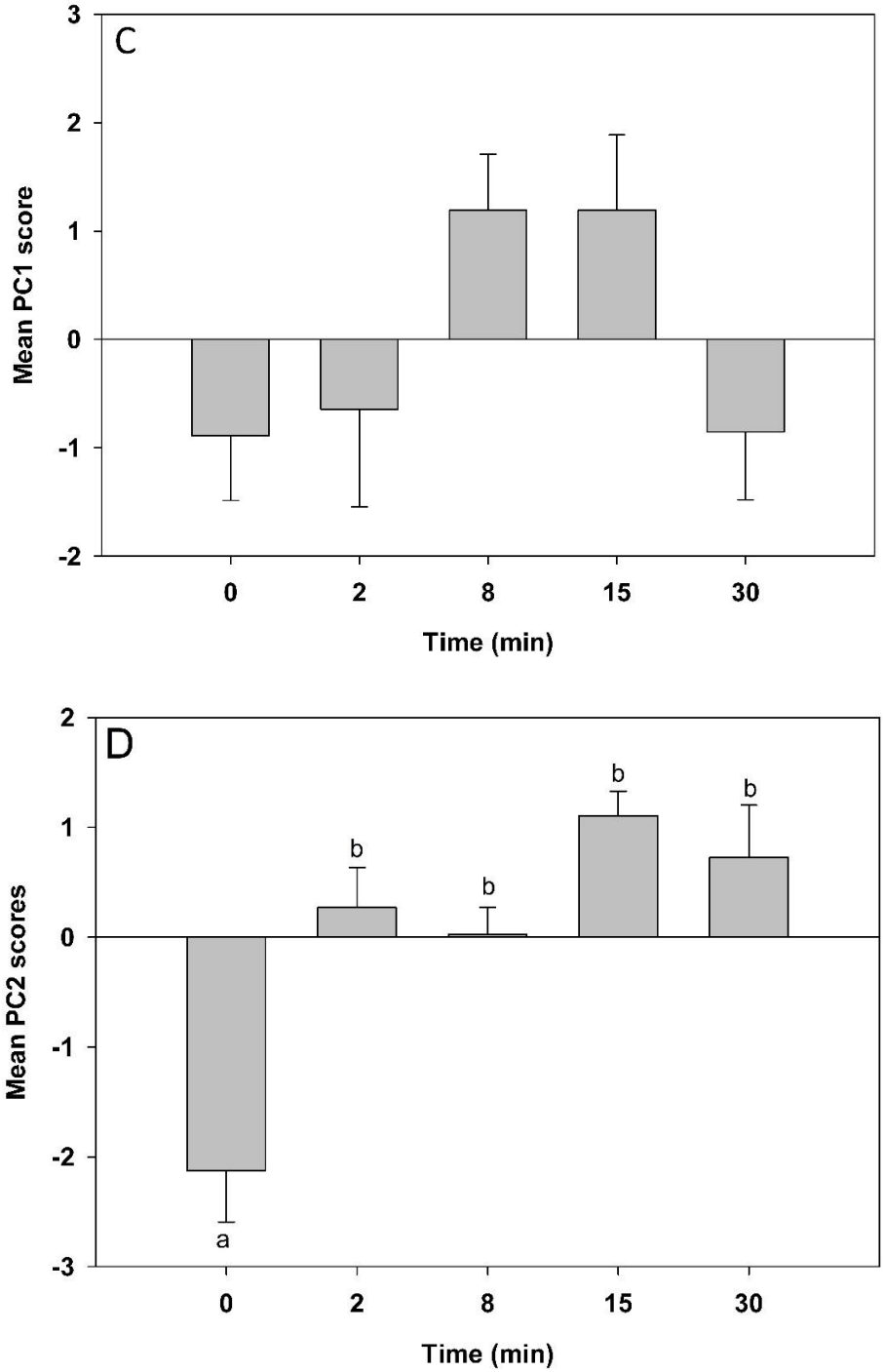
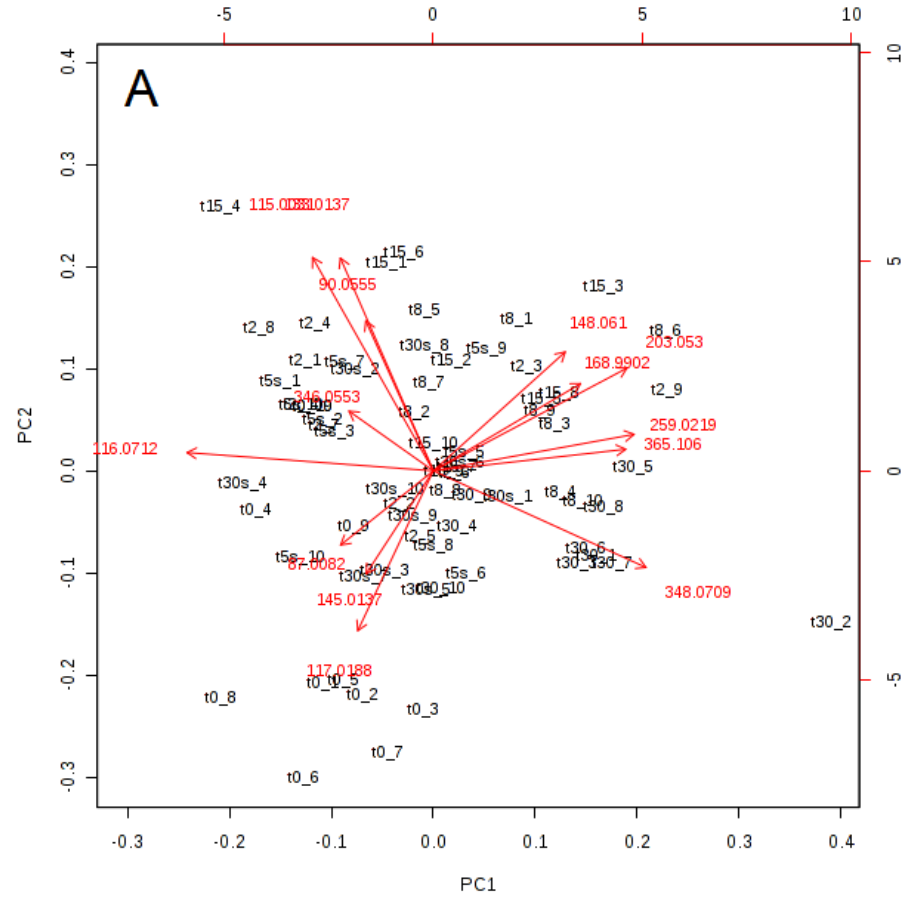


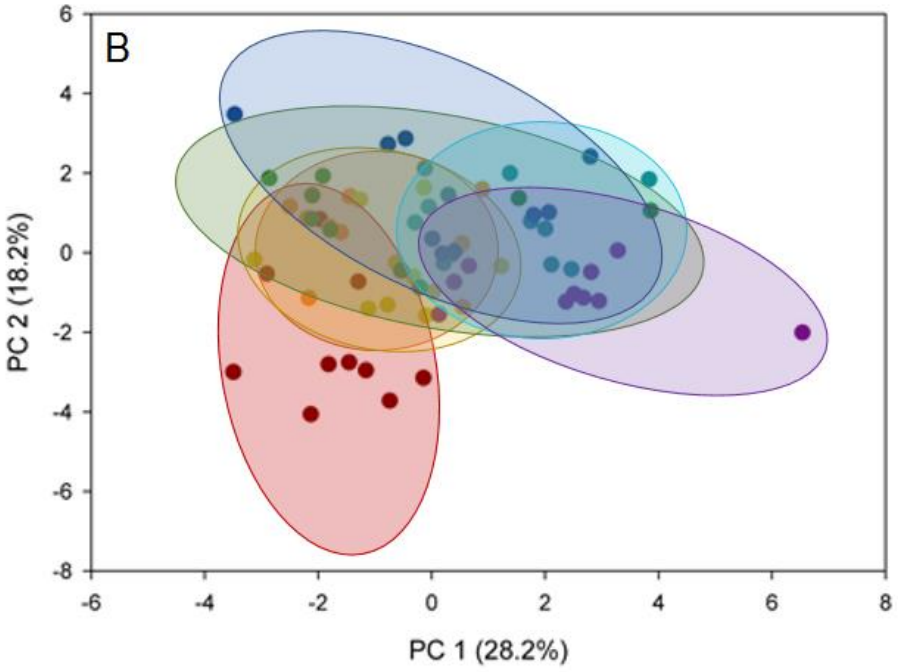
Figure 1. Biplot and scores plot for the first flight experiment thoracic metabolites. **A.** Biplot for the first flight experiment, vectors correspond to the masses of metabolites. **B.** Scores plot for metabolite profiles of individual bees at each time point. Colours of clusters correspond to flight times (red = 0min, orange = 2min, yellow = 8min, green = 15min, blue = 30min). **C.** Changes in mean PC1 scores over time ($p = 0.049$) and **D.** changes in the mean PC2 scores ($p < 0.001$) over time for the first flight experiment.

In the second thoracic dataset, 14 of the detected metabolites were reduced to the first two principal components, accounting for 46.4% of the variation. In the biplot (Fig. 2A), PC1 is dominated by trehalose, G6P and F6P, as well as proline, similarly to what was observed with the previous dataset. The vector for proline extends in the opposite direction because the response is opposite to what occurs in the other metabolites: proline decreases steadily over the 30 minutes, while trehalose and G6P and F6P increase slightly towards the end. Unlike the last dataset, the vector for AMP is angled further from trehalose and G6P/F6P, so the response profiles are slightly different. The vector for glucose and fructose (203.053) is also angled closely to that of trehalose, so they are correlated and the response to time of flight is similar, but trehalose shows a greater increase at 30 seconds than glucose. These metabolites collectively contribute to the most variation seen across PC1, and it appears that carbohydrates and glycolytic intermediates have a response profile opposite to that seen in proline – the fuels are being used differently. PC2 appears to be dominated mainly by fumarate (115.0031), alanine (90.0555), and malate (133.0137) in the positive direction, while α-ketoglutarate, pyruvate, and succinate contribute most to variation in the opposite direction. The reaction profiles are not completely opposite to each other as the vectors are not 180° apart, and similarly to the first dataset, their vector lengths are shorter than those across PC1, indicating less of an effect of time. For example, all of the PC2-dominant metabolites eventually decrease in concentration after a 30 minute flight, but their profiles can be grouped by when this decrease occurs. Fumarate, alanine, and malate remain at higher concentrations throughout the duration of flight with a sharp decrease at the end. Conversely, α-ketoglutarate, pyruvate, and succinate show a large decrease much earlier on in flight, remaining steady until the end of the 30 minutes.

In the scores plot (Fig. 2B), individuals are represented by points and are grouped by flight time. The shifts in the clusters at the different time points are indicative of the overall metabolite profile changes within individuals. In this dataset, it appears that the most variation in metabolic profiles among individuals occurs at 2, 15, and 30 minutes of flight. It is clear that there is a large change from 0 to 2 minutes, as the cluster noticeably shifts upwards. Since the biplot shows proline, trehalose, G6P, and F6P contributing to the most variation, there may be a large difference between these metabolites occurring between these two time points. There is a lot of overlap between the 5 and 30sec clusters, and 2 and 8min, suggesting that less change occurs between these time points overall, despite variation among individuals. The most separation occurs between the data describing 0 and 15 min, and 0 and 30-minute clusters, indicative of the large differences seen between starting and ending concentrations of many metabolites.

Similarly, the changes in the mean scores of PC1 and PC2 for the second flight experiment were plotted throughout flight time. ANOVA tests show significant change occurred among the scores ($F_{6,63} = 8.615$, $p < 0.001$, Fig. 2C). Increases in score were seen between almost all time points and 30 min ($p \leq 0.001$, $p = 0.042$ between 15 and 30 min), indicating a gradual increase in score over time. This change corresponds to the gradual separation of clusters in the scores plot, such as the evident shift from rest to 8, 15, and 30 min. These results highlight the minimal overlap at the end of flight. The PC2 scores change as well ($F_{6,63} = 13.319$, $p < 0.001$, Fig. 2D), with most of the increases occurring between rest and 15 min ($p \leq 0.002$). As in PC1, a large shift occurs in a different subset of metabolites in the overall profile at the onset of flight, but we see a return to the starting coordinates along the PC2 axis.


A
PC1
PC2
90.0555
148.061
203.053
168.9902
346.0553
259.0219
365.106
116.0712
87.0082
145.0137
348.0709
117.0188


B
PC 1 (28.2%)
PC 2 (18.2%)

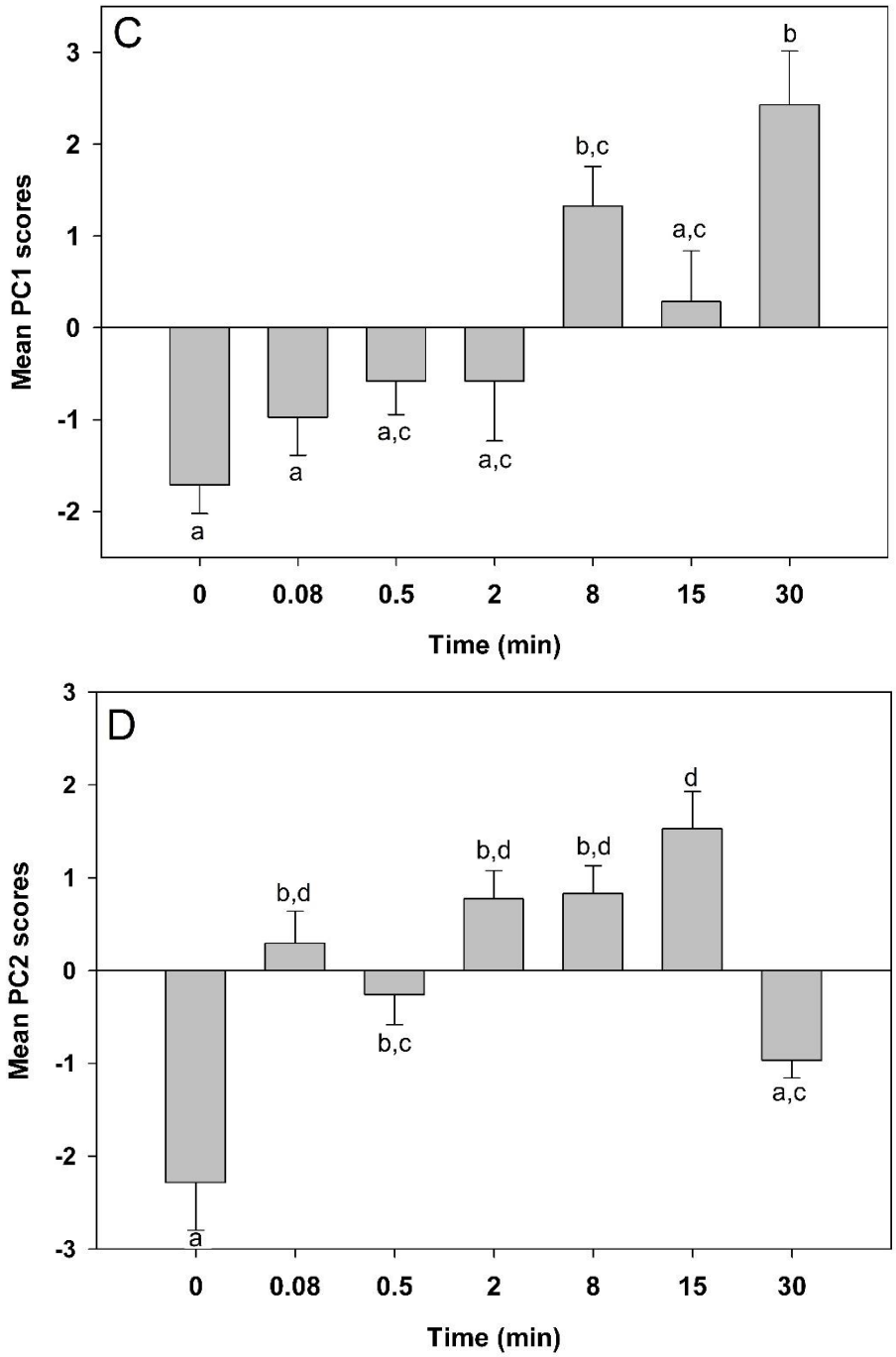


Figure 2. A. The biplot for the second thoracic dataset. Vectors correspond to the masses of metabolites. **B.** Scores plot for collective metabolite intensities of individual bees at each time point in the second dataset. Colours of clusters correspond to flight times (red = 0min, yellow = 5sec, orange = 30sec, green = 2min, light blue = 8min, dark blue = 15min, purple = 30min). **C.** Changes in mean PC1 scores over time ($p < 0.001$) and **D.** changes in the mean PC2 scores over time ($p < 0.001$) for the second flight experiment.

Proline and amino acid metabolism in tissues and hemolymph

In the first thoracic flight experiment, there was no significant change over time in proline concentration ($p > 0.05$; Fig. 3A). The addition of shorter times in the second dataset revealed that proline concentration was changing ($F_{6,63} = 6.464$, $p < 0.001$, Fig. 3B). Pair-wise comparisons demonstrated that these changes were decreases, predominantly occurring between t = 0 or t = 0.08 and latter time points ($p \leq 0.005$ and $p < 0.05$, respectively). There is a clear decline in overall concentration between rest (0 min) and 30 min of flight, with a decrease of 2.746 μmol/g of tissue of proline. Conversely, in both datasets proline remains at the same stable concentration from 2 to 30 minutes. Similar to proline, thoracic alanine concentration did not change during flight with sparser timepoints ($p > 0.05$, Fig. 3C). When shorter flight times were included, alanine concentration did change ($F_{6,63} = 8.94$, $p < 0.001$, Fig. 3D). Post-hoc analysis and pairwise comparisons revealed decreases in concentration, occurring predominantly between the shortest time points and those exceeding 8 min ($p < 0.03$), with the largest change being a 33% decrease in concentration between 0.08 min and 30 min. As seen in the other amino acids, glutamate quantities did not change in the first dataset ($p > 0.05$, Fig. 3E). In the second dataset, glutamate concentration changed over time ($F_{6,54} = 3.873$, $p = 0.003$, Fig. 3F). In contrast, the changes were increases. They predominantly occurred between the shorter 0.5 min flight to 8, 15, and 30 min ($p = 0.035$, 0.011, and 0.002, respectively). Similarly, increases in concentration occurred from 2 to 15 and 30 min ($p = 0.026$, $p = 0.004$).

In the hemolymph, proline did not show a significant change in concentration throughout the duration of flight ($p > 0.05$, Fig. 4A). Similarly, alanine and glutamate intensity did not change over time ($p > 0.05$, Fig. 4B and C). In abdominal homogenates, proline quantities did not change in concentration after 30 min of flight ($p > 0.05$, Fig. 4D). However, there was a significant

decrease in alanine concentration from 0 to 30 minutes of flight ($F_{1,18}$ = 5, p = 0.042, Fig. 4E), from 1.362±0.116 μmol/g tissue to 1.068±0.062 μmol/g tissue. Glutamate did not change in the abdomen either ($p > 0.05$, Fig. 4F).

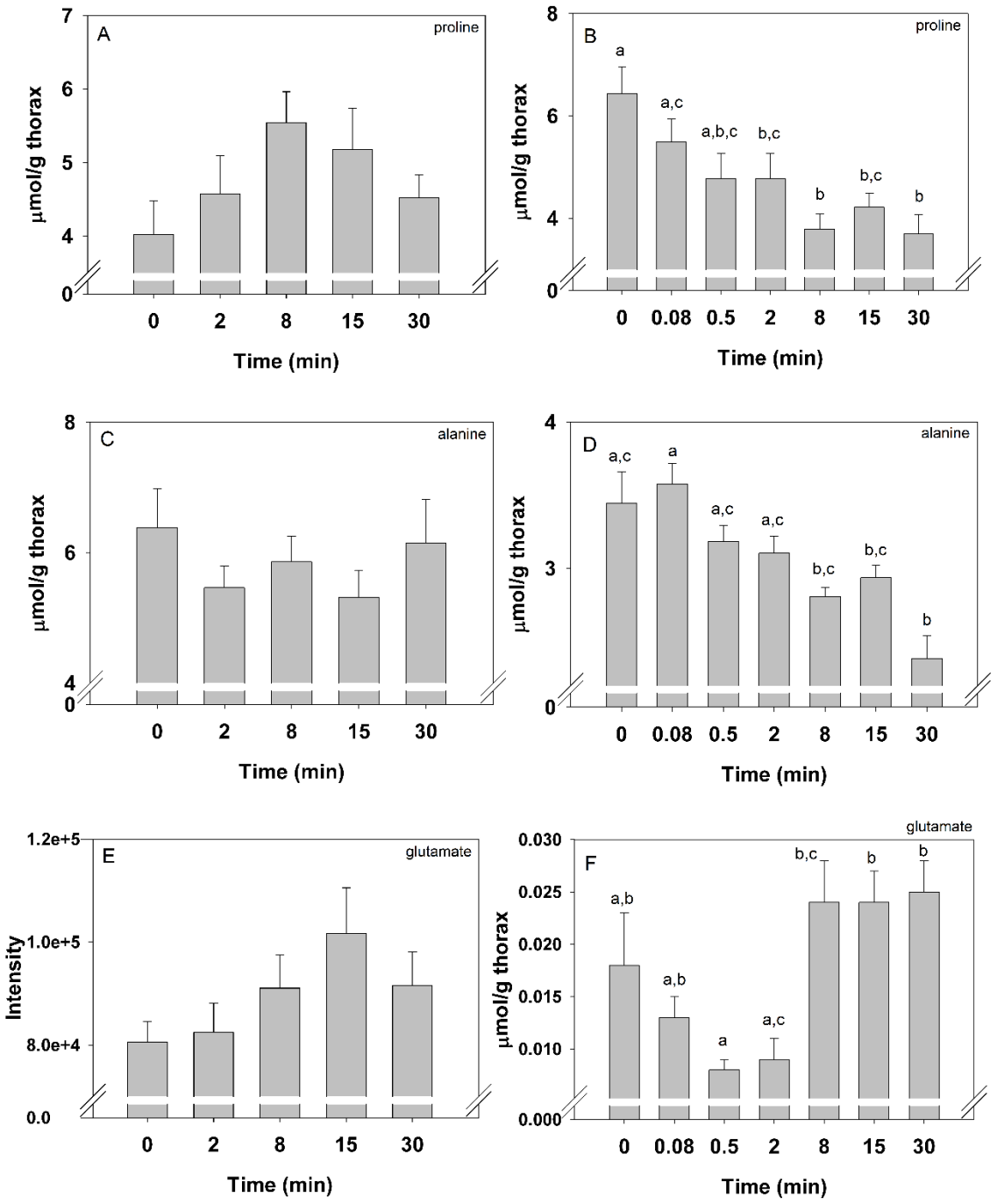


Figure 3. Changes in the concentrations of amino acids in thoracic tissues, expressed as µmol/g tissue. **A.** Proline concentration in the first (p = 0.162) and **B.** second dataset (p < 0.001). **C.** Alanine concentration in the first (p = 0.525) and **D.** second dataset (p < 0.001). **E.** Glutamate intensity in the first thoracic dataset (p = 0.172) and **F.** glutamate concentrations in the second dataset (p = 0.003). Bars with different letters are significantly different.

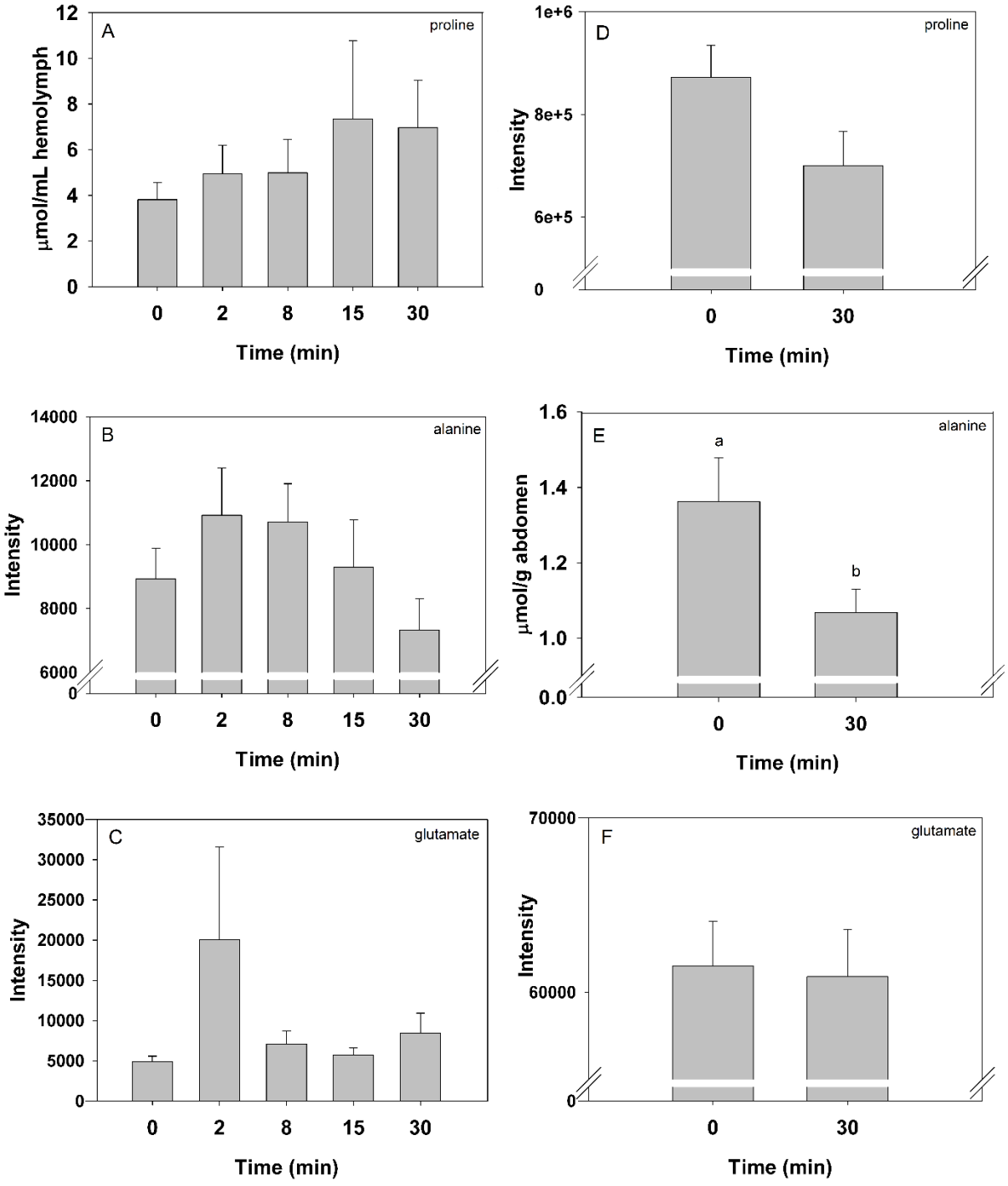


Figure 4. Changes in the concentration of amino acids in the hemolymph and abdominal homogenate. **A.** Proline in hemolymph (p = 0.874), **B.** alanine in hemolymph (p = 0.255), and **C.** glutamate in hemolymph. **D.** Change in abdominal proline (p = 0.074), **E.** alanine (p = 0.042), and **F.** glutamate (p = 0.871).

Trehalose, glycogen, and carbohydrate metabolism

Even at more widespread time points in the first flight experiment, the concentration of trehalose in the thorax was found to be changing significantly ($F_{4,45} = 4.696$, $p = 0.003$, Fig. 5A). Post-hoc tests show that there is a decrease in trehalose from rest to 8 min ($p = 0.019$), followed by an increase by 30 min ($p = 0.011$), creating a U-shaped curve. When shorter flight times were incorporated, trehalose concentration also changed ($F_{6,63} = 3.703$, $p = 0.003$, Fig. 5B). Large increases occur from 0.08 to 0.5 min ($p = 0.017$) and 0.08 to 30 min ($p = 0.049$). In contrast, the U-shape occurs a bit later, and is noticeable from 0.08 to 30 min rather than from rest. In the hemolymph, trehalose concentration did not appear to change ($p > 0.05$, Fig. 5C). In contrast, abdominal concentration of trehalose increased after 30 min, by approximately 0.085 µmol/g abdomen ($F_{1,18} = 7.552$, $p = 0.013$, Fig. 5D). In parallel to overall trehalose content, abdominal glycogen concentration decreased after a 30 minute flight. It decreased by more than half of its total quantity, from approximately 0.288µmol/g abdomen to 0.123µmol/g abdomen ($F_{1,8} = 8.804$, $p = 0{,}018$, Fig. 5E).

In addition to trehalose, the carbohydrate fuels glucose and fructose were detected. In the first dataset, the two metabolites were detected as a combined peak intensity and there was no change in their collective quantity ($p > 0.05$, Fig. 6A). Peak intensities were also collected in the second dataset. The addition of shorter flight times resulted in similar patterns seen in trehalose, where a U-shape occurred between 0.08 and 30 min. Overall, the two metabolites increased over time ($F_{6,63} = 3.322$, $p = 0.007$, Fig. 6B). In the abdomens, glucose and fructose increased by about 60% after 30 minutes of flight ($F_{1,18} = 9.765$, $p = 0.006$, Fig. 6C).

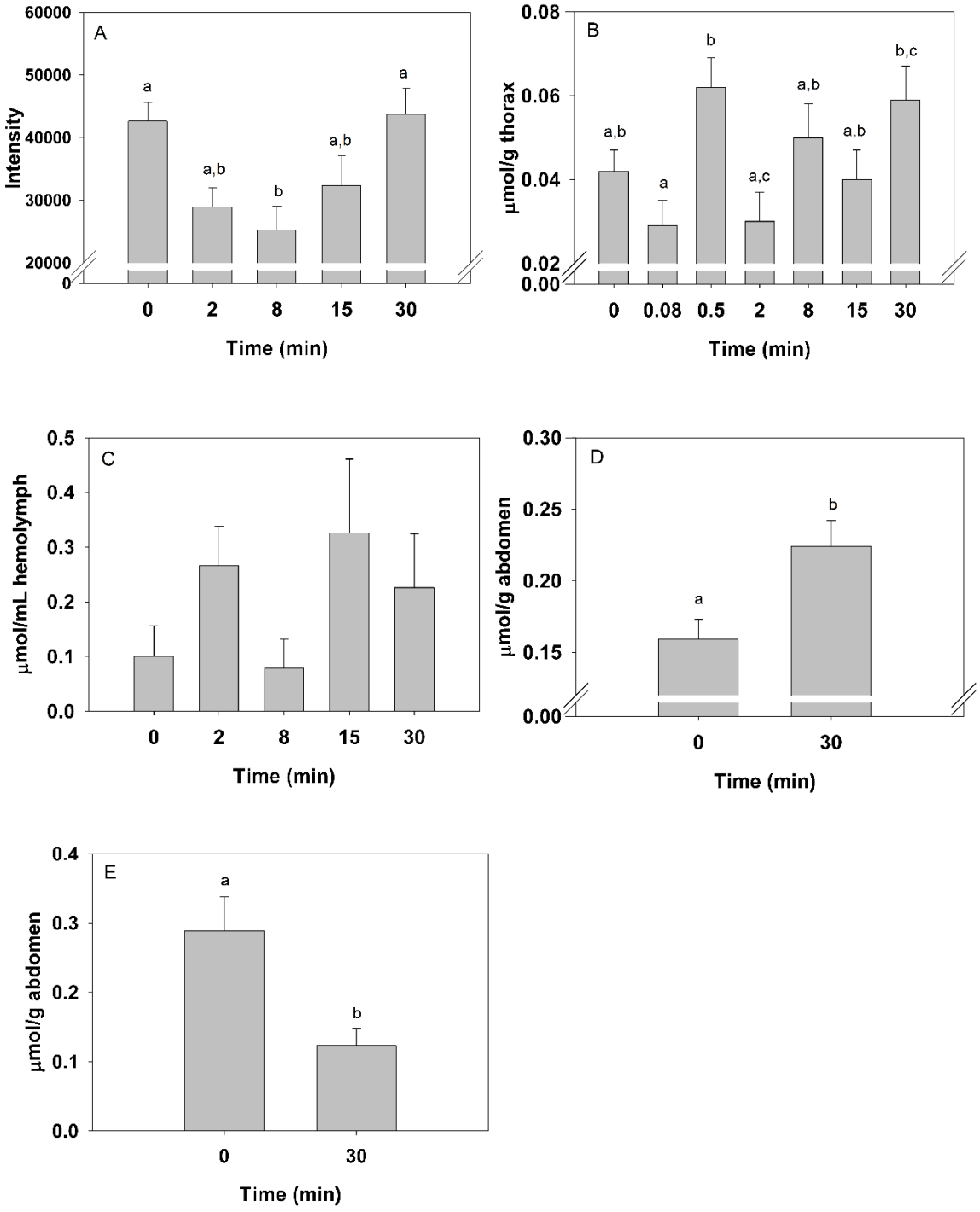


Figure 5. Changes in trehalose and glycogen content during flight. **A.** Trehalose in the first thoracic dataset ($p = 0.003$) and **B.** in the second thoracic dataset ($p = 0.003$). **C.** Changes in trehalose concentration in the hemolymph ($p > 0.05$) and **D.** in the abdomen ($p = 0.003$). **E.** Change in abdominal glycogen after 30 min of flight ($p = 0.018$).

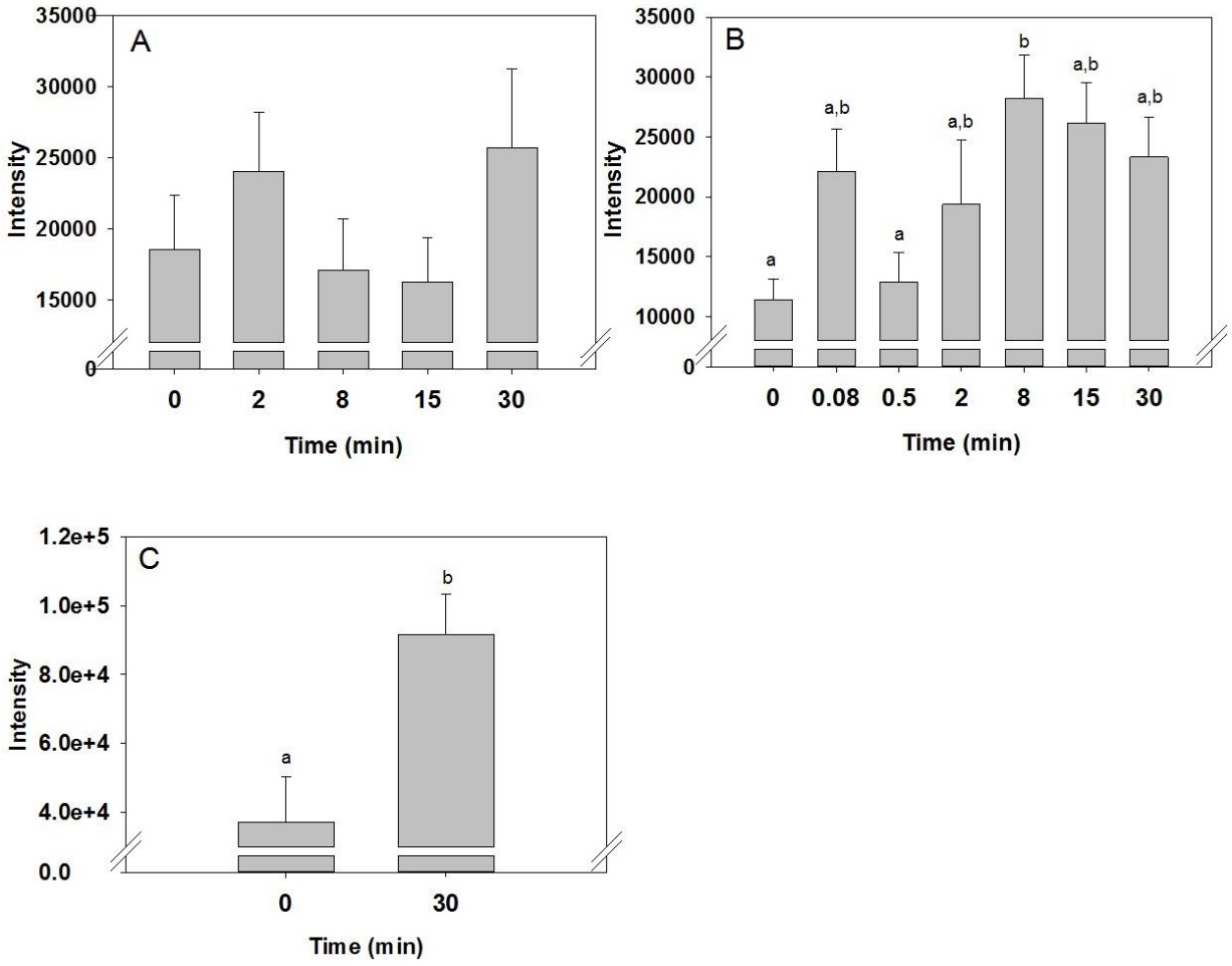


Figure 6. Changes in glucose and fructose ion intensities in thoracic tissues. **A.** Ion intensity of glucose and fructose in first thoracic dataset (p = 0.383, n = 10) **B.** Ion intensity of glucose and fructose from the second thoracic dataset (p = 0.007, n = 10). **C.** Change in abdominal glucose and fructose (p = 0.006, n = 10).

Glycolytic intermediates

Glycolytic intermediates that were detected in tissue samples included G6P, F6P, DHAP, G3P, 3PG, and pyruvate. In the first dataset, there was a significant change in the quantity of G6P and F6P ($F_{4,45} = 6.389$, $p < 0.001$, Fig. 7A). Decreases occurred from 0 to 8 and 0 to 15 min ($p = 0.033$, $p = 0.016$, respectively) while increases occurred during the latter half of flight, from 8 to 30 min and 15 to 30 min ($p = 0.004$, $p = 0.002$, respectively). In the second dataset, the addition of shorter flight times did not reveal any changes at the start of flight in either G6P or F6P. Rather, the changes occurred in the second half of flight, like in the first dataset. G6P changed significantly ($F_{6,63} = 5.717$, $p < 0.001$, Fig. 7B), with increases from 0.08 to 8, 15, and 30 minutes ($p = 0.01$, 0.012, and $p < 0.001$, respectively) and from 2 to 30 minutes ($p = 0.004$). F6P also increased significantly during flight ($F_{6,63} = 5.717$, $p < 0.001$, Fig. 7C), within the same time frames seen in G6P. Neither of these metabolites changed in the abdomen ($p > 0.05$).

The combined peak intensity for DHAP and G3P in the first flight experiment showed no change ($p > 0.05$, Fig. 7D). In the second experiment, concentrations were determined for both metabolites. Similarly, neither DHAP nor G3P were changing ($p > 0.05$, Fig. 7E and F). In the abdomens, DHAP and G3P ion intensity appeared to increase from barely detectable levels to just surpassing the detection threshold by 30 minutes. Although it was possible to note that both compounds were increasing over time ($p = 0.003$), it is likely that these metabolites were not present in large amounts in the abdomen. 3PG did not change between widespread flight times in the first dataset ($p = 0.13$, Fig. 8A). In the second dataset, there were several decreases in concentration ($F_{6,63} = 6.08$, $p < 0.001$, Fig. 8B), occurring only between 0 minutes and all remaining time points ($p < 0.05$), excluding 0.08 min. 3PG was also not detected in the abdomen at sufficient quantities for analysis.

Finally, pyruvate was found to be changing in the first dataset ($F_{4,45} = 9.5$, $p < 0.001$, Fig. 8C). There were large decreases in quantity from 0 to 2 min ($p = 0.006$), followed by increases towards the second half of flight, from 15 min to 30 min ($p = 0.024$, respectively). Similarly, this metabolite changed in a U-shaped pattern as well. With the addition of shorter flight times, pyruvate also showed changes in concentration ($F_{6,50} = 13.69$, $p < 0.001$, Fig. 8D). However, the increase in the second half of flight seen in the first dataset was not observed. The changes all manifested as decreases, from the shortest to the longest flight times, with the largest overall decrease of 0.704 µmol/g tissue occurring from the start to the end of flight ($p = 0.011$). The concentration of pyruvate did not change in the abdomen ($p > 0.05$, Fig. 8E).

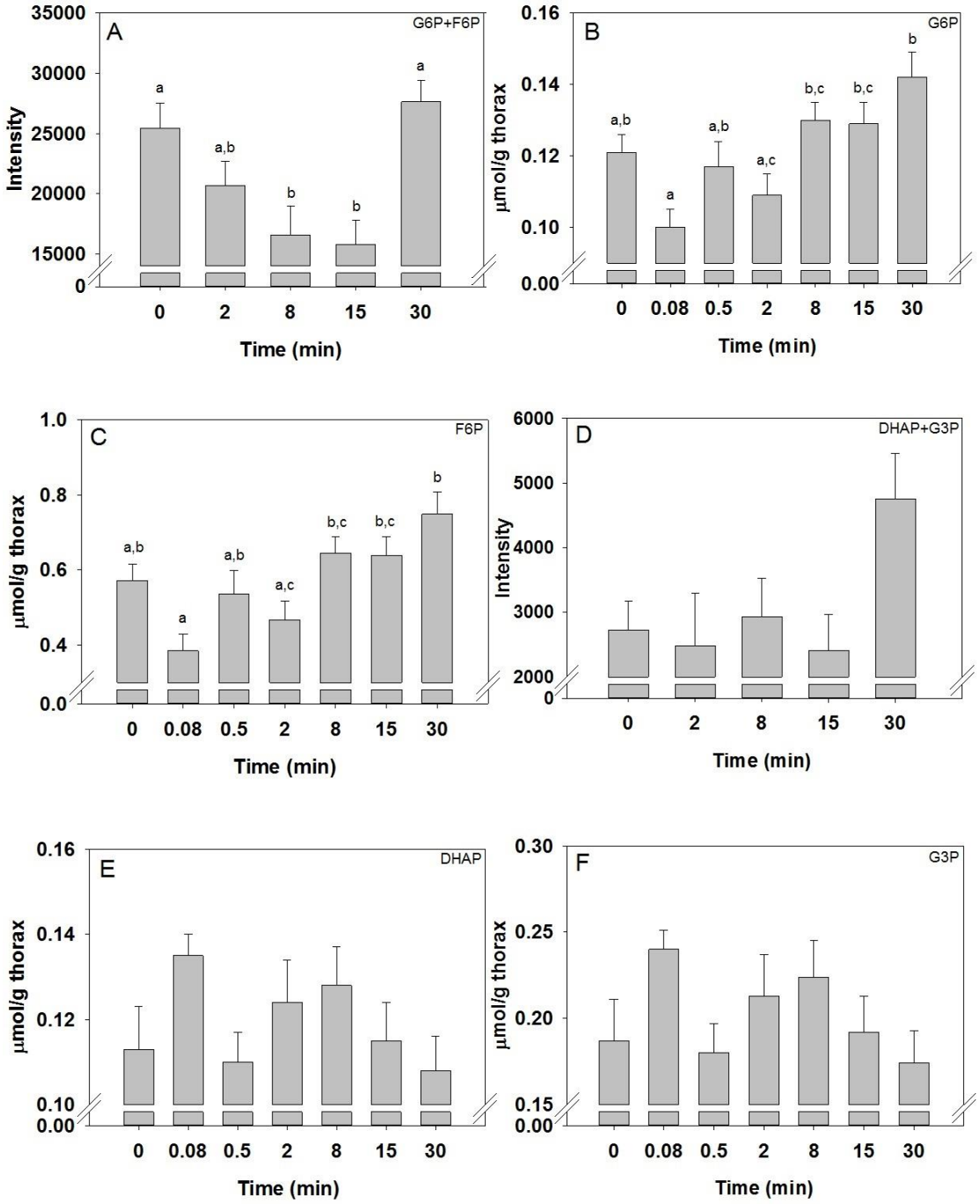


Figure 7. Change in G6P, F6P, DHAP, and G3P glycolytic intermediates in thoracic tissues. **A.** Combined peak intensities of G6P and F6P in first thoracic dataset ($p < 0.001$, n = 10). **B.** G6P concentration in the second thoracic dataset ($p < 0.001$, n = 10), and **C.** F6P in second thoracic dataset ($p < 0.001$, n = 10). **D.** DHAP and G3P combined peak intensities in the first thoracic dataset ($p = 0.067$, n = 10). **E.** DHAP concentration in second thoracic dataset ($p = 0.199$, n = 10) and **F.** G3P from the second thoracic dataset ($p = 0.199$, n = 10).

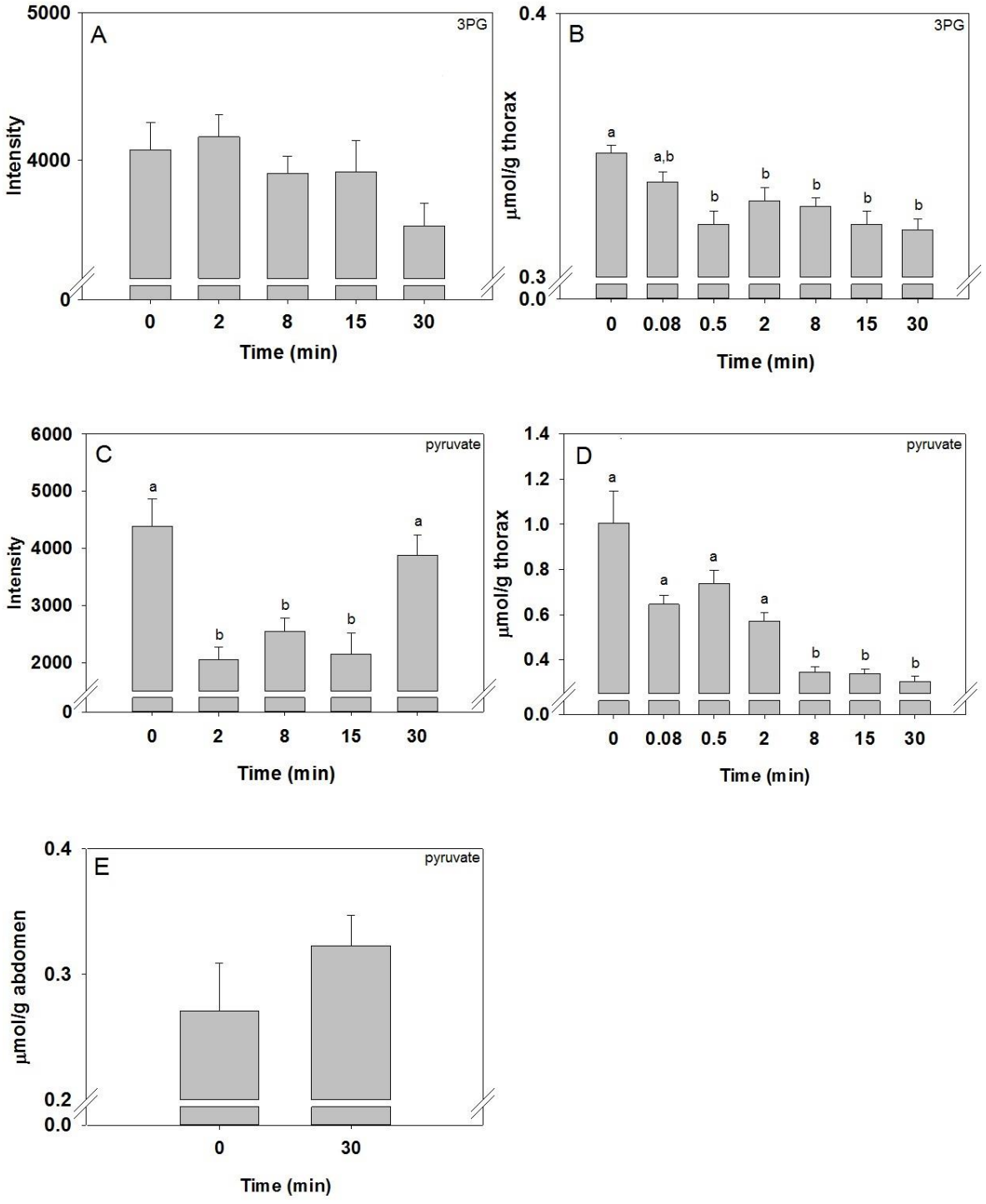


Figure 8. Change in glycolytic intermediates 3PG and pyruvate in thoracic tissues. **A.** 3PG peak intensities from the first thoracic dataset ($p = 0.13$, n = 10) and **B.** 3PG concentrations from the second thoracic dataset ($p < 0.001$, n = 10). **C.** Pyruvate intensities from the first thoracic dataset ($p < 0.001$, n = 10), and **D.** pyruvate concentrations in second thoracic dataset ($p < 0.0010$). **E.** Pyruvate concentration in abdomens ($p = 0.264$).

Krebs cycle metabolites and oxidative phosphorylation components in tissues

Detected Krebs cycle intermediates included succinate, malate, α-ketoglutarate, and fumarate. In the first dataset, succinate intensity was changing significantly ($F_{4,45}$ = 2.766, p = 0.039, Fig. 9A), with a decrease from 0 min to 2 minutes of flight (p = 0.057). Similarly, succinate concentration changed when shorter flight times were added ($F_{6,60}$ = 24.512, p < 0.001, Fig. 9B). However, the concentration of succinate at rest was markedly higher than at all other times. This resulted in significant decreases from rest to all other time points ($p \leq 0.001$). In the abdomen, succinate concentration showed no change ($p > 0.05$, Fig. 9E). Malate quantities in the first dataset did not change ($p > 0.05$, Fig. 9C), though it did change during shorter flight times ($F_{6,63}$ = 5.694, $p < 0.001$, Fig. 9D). Concentrations remained stable until 30 minutes of flight, at which point there was a decrease. Malate did not change significantly in the abdomen (p = 0.079, Fig. 9F).

A-ketoglutarate concentration changed between longer flight times ($F_{4,33}$ = 2.935, p = 0.035, Fig. 10A), decreasing in the first half of flight and remaining stable. Shorter flight times also caused changes in the metabolite ($F_{6,63}$ = 20.091, $p < 0.001$, Fig. 10B). As seen in succinate, the amount of α-ketoglutarate was notably larger at rest than at all other time points, resulting in a sharp decrease between rest and 5 seconds ($p < 0.005$), followed by stability. In abdomens, α-ketoglutarate decreased from rest to the end of a 30 min flight ($F_{1,18}$ = 6.707, p = 0.018, Fig. 10E). The final Krebs intermediate detected was fumarate, which changed significantly in both flight experiments. In the first ($F_{4,45}$ = 13.833, $p < 0.001$, Fig. 10C), there were decreases in fumarate from rest to all other time points ($p \leq 0.008$), as well as decreases from 2 and 8 min to 15 min (p = 0.009, p = 0.018, respectively). The second experiment ($F_{6,62}$ = 6.051, $p < 0.001$, Fig. 10D) revealed a sharp decrease at 30 minutes of flight, though the decreases were significant at all time points ($p < 0.05$). The abdominal concentration of fumarate did not change ($p > 0.05$, Fig. 10F).

AMP and cyclic GMP make up the detected components of the oxidative phosphorylation system. Using longer flight times, AMP did not change throughout flight ($p > 0.05$, Fig. 11A). When shorter time points were added, the concentration appeared to be changing ($F_{6,63} = 2.732$, $p = 0.04$, Fig. 11B), but only between two timepoints. There was an increase in concentration from 0.5 min to 30 min ($p = 0.044$). In the abdomens, there was no significant change in the amount of AMP ($p > 0.05$, Fig. 11E). Similarly, cGMP was not changing between longer flight times ($p > 0.05$, Fig. 11C), though it showed change in the second dataset ($F_{6,63} = 2.814$, $p = 0.017$, Fig. 11D). There was an increase in concentration also from 0.5 min, to 15 min ($p = 0.048$). cGMP was not detected in the abdomens at high enough intensities to be included in analysis.

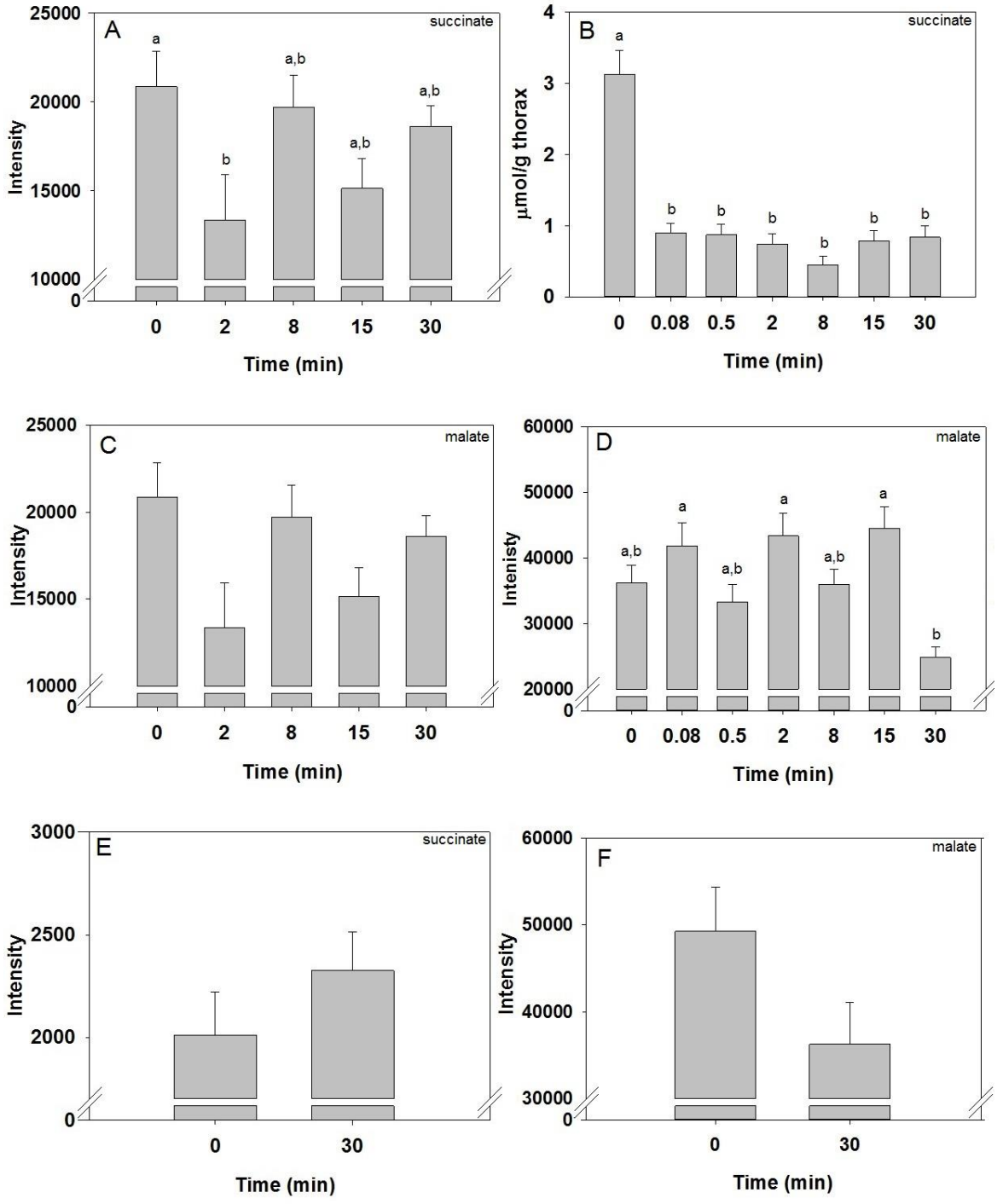


Figure 9. Krebs cycle intermediates succinate and malate in thoracic tissues. **A. S**uccinate content in the first thoracic dataset (p = 0.039, n = 10) and **B.** concentration in the second dataset (p < 0.001) **C.** changes in malate in the first thoracic dataset (p < 0.001, n = 10) **D.** Changes in thoracic malate from the second dataset (p = 0.139, n = 10). **E.** Changes in abdominal succinate content (p = 0.282, n = 10) and **F.** changes in abdominal malate (p = 0.070, n = 10).

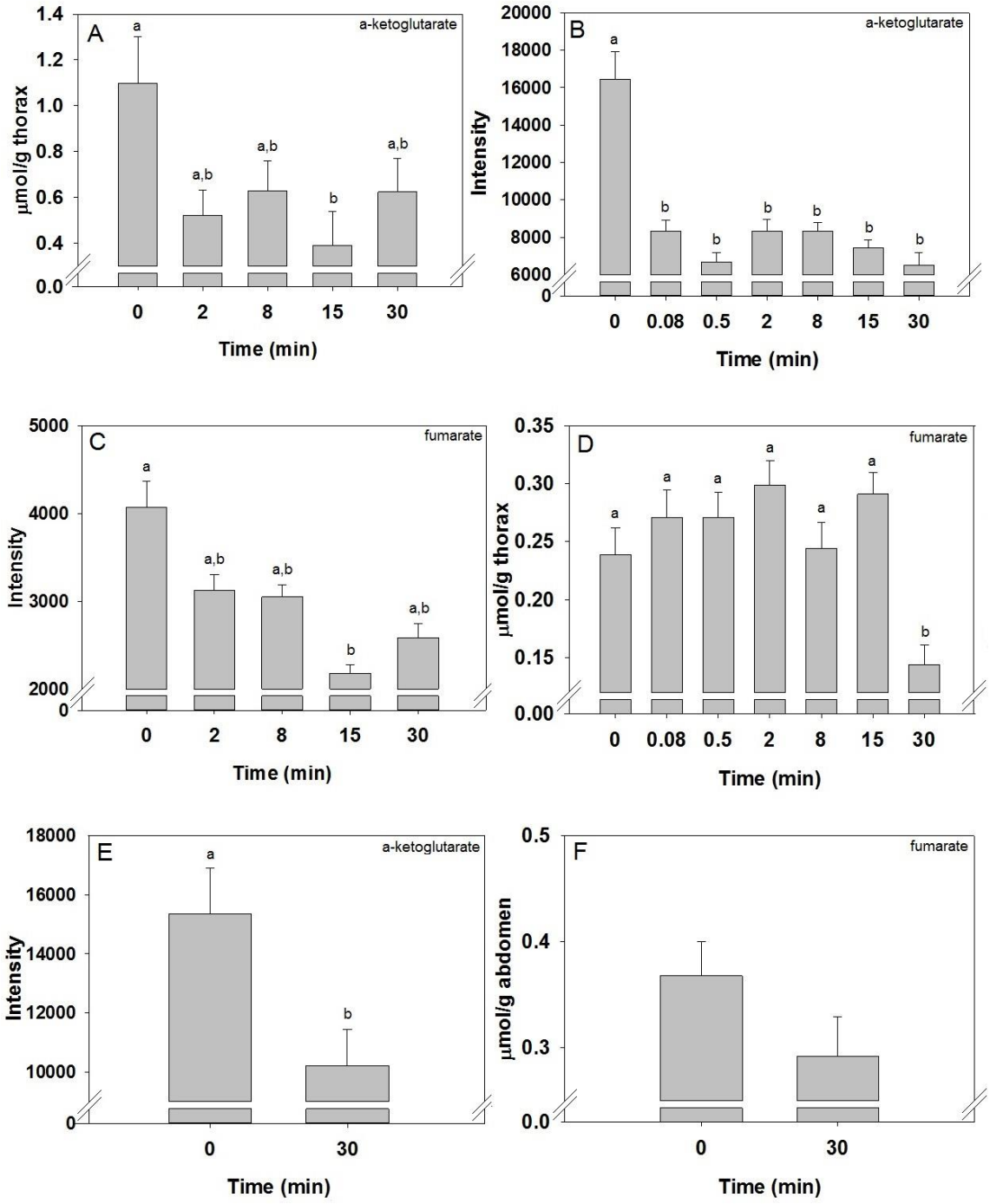


Figure 10. Krebs cycle intermediates α-ketoglutarate and fumarate in thoracic tissues. **A.** Change in α-ketoglutarate concentration from the first thoracic dataset (p = 0.035) **B.** Change in α-ketoglutarate from the second thoracic dataset (p < 0.001, n = 10). **C.** Fumarate concentration in the first thoracic dataset (p < 0.001). **D.** Change in fumarate in the second thoracic dataset (p < 0.001). **E.** Change in abdominal α-ketoglutarate content (p = 0.018, n = 10). **F.** Change in abdominal fumarate concentration (p = 0.137, n = 10).

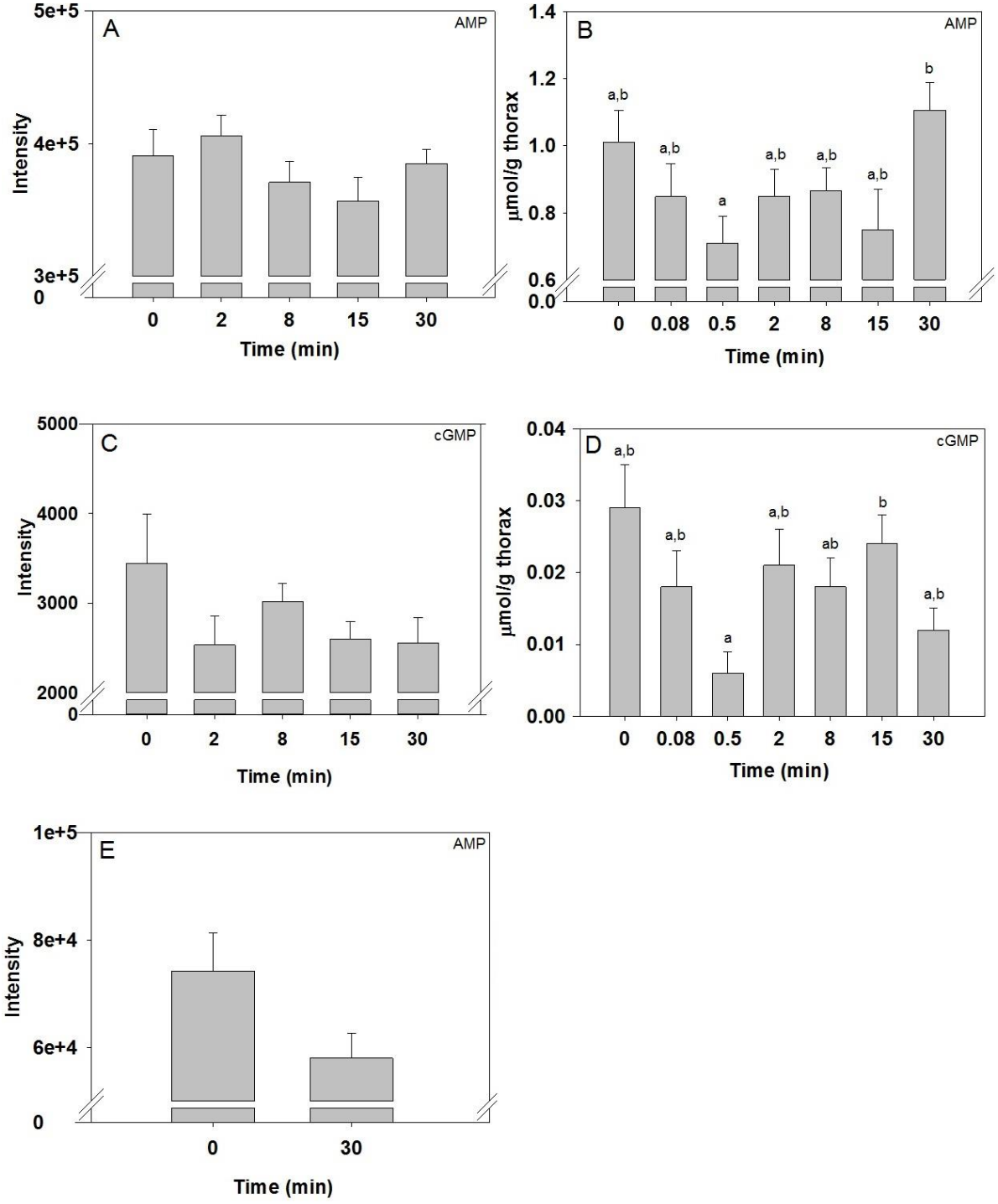


Figure 11. Changes in the components of oxidative phosphorylation in tissues. **A.** AMP content in the first thoracic dataset (p = 0.281, n = 10) and **B.** the second thoracic dataset (p = 0.004, n = 10). **C.** cGMP in the first thoracic dataset (p = 0.257, n = 10) and **D.** cGMP in the second thoracic dataset (p = 0.017, n = 10). **E.** Abdominal AMP content (p = 0.074, n = 10).

Hemolymph metabolites

Although several metabolites were detected in the hemolymph samples, ultimately there was minimal change occurring in this sample type throughout the flights, with the exception of fumarate ($F_{4,45} = 9.857$, $p < 0.001$, Fig. 12). There were increases in quantity from shorter flight times to the end of a 30 minute flight.

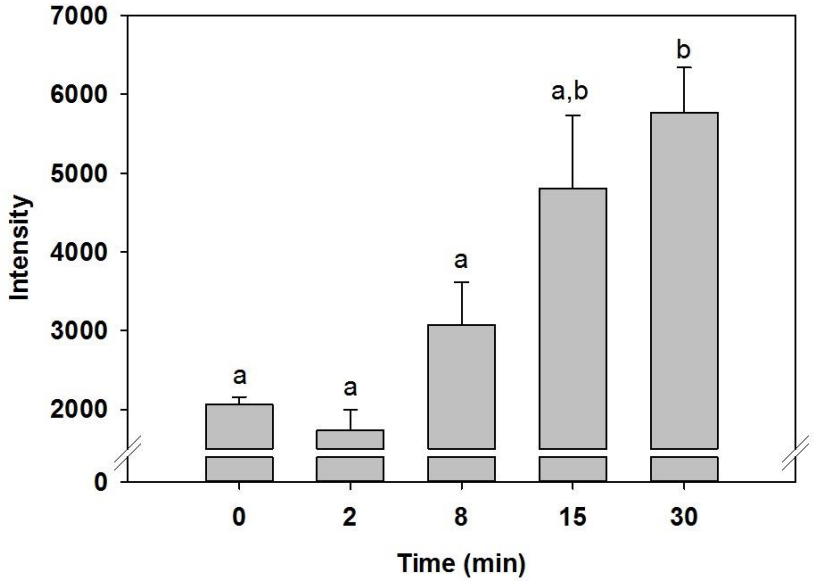


Figure 12. Change in hemolymph fumarate content ($p < 0.001$, n = 10).

Discussion

Proline metabolism during bumblebee flight

Various substrates can be used in order to fuel the activities of animals, including carbohydrates, proteins, and lipids. Although the ways these substrates are used is better documented in groups like vertebrates, insects are unique due to their unusual ability to substantially use the amino acid proline as a fuel. However, there is variation in proline use across insects as well. In the order Hymenoptera, for example, honeybees use carbohydrates almost exclusively, while sister species like bumblebees (*Bombus impatiens*) can oxidize proline at high rates (Teulier et al., 2016). Like honeybees, bumblebees were thought to use carbohydrates as a substrate for activities like flight, though Carter et al. (2006) proposed that plants use proline as a metabolic reward for pollinating insects. Given that proline oxidation occurs in isolated bumblebee flight muscle tissues, it is possible that proline is used as a substrate for flight. This study investigated changes in metabolite content during flight through generating metabolite profiles at key metabolic transition points during flight progression. Ultimately, we found little evidence that proline is a substantial fuel for bumblebee flight, due to the lack of alanine accumulation and overall decrease in proline. This raises the question of why this species has the ability to oxidize proline to such an extent. Through metabolic profiling it was also evident that although metabolite content is tightly regulated, global changes over time can be detected, especially at shorter flight times.

The role of proline oxidation during flight is well documented in many insect species, and many are capable of using proline as a co-substrate with carbohydrates to power flight. Among those species, some opt to use it transiently as a “sparker”, like the dipteran blowfly (Sacktor and

Wormser-Shivat, 1966). In this case, proline is used to supplement Krebs cycle intermediates at the onset of flight. It has been demonstrated that when proline and pyruvate are provided to the blowfly mitochondria, there is an increase in Krebs cycle intermediate content (Johnson and Hansford). Coleopteran species such as Colorado potato beetle (Weeda et al., 1979, Weeda et al., 1980a) can use proline to a larger extent as a co-substrate with carbohydrates, during flight and in mitochondrial preparations. The African fruit beetle similarly oxidizes proline simultaneously with carbohydrates, and proline and pyruvate led to the highest oxidation rates in isolated flight muscle mitochondria (Zebe and Gäde, 1999). Proline can be oxidized either partially or completely, and is converted into glutamate that enters the Krebs cycle as α-ketoglutarate via trans- or deamination. Partial oxidation requires the action of alanine aminotransferase (AAT) to create α-ketoglutarate for acetyl-CoA oxidation through the Krebs cycle (Zebe and Gäde, 1993). In turn, AAT can be used to convert accumulated alanine back into proline in an equimolar fashion. In contrast, the complete oxidation of proline requires the activity of glutamate dehydrogenase, producing ammonia as a result (Gäde and Zebe, 1999; Storey, 1985).

Given all the ways insects can metabolize proline, we initially hypothesized that proline would be used in conjunction with carbohydrates, and that both fuels would decrease at the onset of flight. In species using both fuels, the thoracic concentrations have been shown to decrease at the onset of flight. For example, Zebe and Gäde (1993) found that in the African fruit beetle, thoracic proline decreases from about 65µmol/g to 16µmol/g, or by about 75%. Glycogen shows a similar decrease. Our findings demonstrate that although thoracic proline decreases during flight, it is not as drastic, and the hemolymph content remains unchanged. There is also no concomitant increase in alanine concentration in either the thorax or hemolymph. Evidently, proline is not used to the same extent in bumblebees as in coleopterans, and certainly not to the same extent as blood-

feeding tsetse flies as they show almost negligible carbohydrate reserves due to their sole use of proline (Bursell, 1981). Bumblebees are likely not undergoing complete proline oxidation either, due to the low glutamate dehydrogenase activity in bumblebee flight muscle tissue (Simoneau and Darveau, unpublished). Its low activity suggests it is not actively used in proline metabolism, or simply exists in lower quantities. Partial oxidation through AAT is therefore the alternative option. However, we also observed a decrease in carbohydrates like trehalose, and saw abdominal glycogen stores deplete over the course of a 30 minute flight. Since Hymenoptera characteristically use carbohydrates in catabolic metabolism, we suspected that proline is simply used to supplement the Krebs cycle as a "sparker", like the blowfly (Childress and Sacktor, 1966), which also relies predominantly on carbohydrates. The concentrations of proline found in bumblebees was much lower than in insects that use proline as a primary fuel, like the Colorado potato beetle and tsetse fly (up to 40 and 150µmol/g in flight muscles, respectively) (Beenakkers et al., 1984; Bursell 1963). Bumblebees do not appear to contain large enough proline stores for it to be used as a major fuel. Instead, the concentration was similar to that found in the blowfly (6µmol/g tissue).

Proline oxidation can be transient and used for short durations to help power take-off and high-speed flight in Hymenoptera and Diptera. For example, in the blowfly, thoracic proline shows dramatic decrease within the first few seconds of flight (Sacktor and Wormser-Shavit, 1966). We saw similar trends in proline metabolism in *B. impatiens*, particularly in our second flight experiment, where changes in thoracic amino acid concentrations were observed at shorter flight times. Within 2 minutes, proline concentration significantly decreased in bumblebee thoraxes. This suggests that proline is used to prime Krebs metabolism because trehalose shows an immense decrease within the first few seconds, and partial proline oxidation can provide the necessary aerobic energy by rapid ATP production (Storey, 1985). Proline may be oxidized immediately as

a substrate because thoracic trehalose is quickly depleted. Meanwhile, glycogen must be mobilized to increase readily available trehalose that subsequently creates glucose. Since glucose is not present in large amounts in hemolymph, it must be derived from the hydrolysis of trehalose, and then phosphorylated with the help of ATP and hexokinase (Nation, 2015). Proline oxidation does not require the input of ATP, so it can supply ATP immediately.

Our experiments included time points reflecting the Coleopteran model of proline metabolism proposed by Zebe and Gäde (1993), and a second experiment was included with shorter flight times, based on the findings of Sacktor and Wormser-Shavit (1996) in the blowfly. The Coleopteran model highlights that after 8 minutes of flight, metabolite levels begin to stabilize and reach a steady-state, with no change in flight performance. We found that this occurs in the three detected amino acids in the bumblebees – alanine and glutamate reach steady state around 8 minutes, while proline does earlier. Alanine and proline both decrease during flight, meanwhile glutamate increases. This is similar to findings in the blowfly, which also undergoes the largest changes at the onset of flight, and achieves steady state later (Sacktor and Wormser-Shavit, 1966). The only difference is that glutamate decreases in the blowfly. The African fruit beetle (*P. sinuata*) demonstrates the same pattern – steady state is achieved in alanine and proline within a few minutes of flight (Zebe and Gäde, 1993). In contrast, alanine does not decrease. This confirms the use of proline as a major fuel of working flight muscle in this coleopteran. The tsetse fly and Colorado potato beetle also show linear, steady decline in proline during flight, paralleled by a fast, plateauing increase in alanine concentration (Hargrove 1967, Beenakkers et al., 1984). In the abdomen of *P. sinuata*, proline concentrations exceed 50µmol/g, and remains relatively unchanged earlier in flight, declining subsequently – however, alanine in the abdomen rises in parallel (Zebe and Gäde, 1993). This suggests that abdominal proline reserves are mobilized and replenished by

alanine that accumulates in flight muscle metabolism. Alanine resynthesizes proline in the fat body by hormonal regulation via the adipokinetic hormone (AKH) family of neuropeptides and the addition of 2 carbons from triglycerides (Candy 1997). In contrast, both abdominal proline and alanine decrease in the bumblebee, suggesting alanine is not accumulating in order to resynthesize proline. These differences highlight that while steady-state is achieved in amino acid metabolism in coleopteran, dipteran, and hymenopteran species, the way it is used is largely determined by whether there is a stoichiometric relationship between alanine accumulation and proline use (Sacktor and Wormser-Shavit, 1966). Since both proline and alanine are exhausted in the bumblebee, proline is not used as a fuel beyond the onset of flight and supplementation of the Krebs cycle. Since neither proline or alanine increase in the abdomens, proline is not immediately resynthesized because it is not required.

Glutamate acts as an intermediate step between proline and alanine. Proline is oxidized by flight muscle mitochondria to generate glutamate at rates that correspond to proline use *in vivo* (Sacktor and Wormser-Shavit, 1966). In isolated mitochondria, the sum of the products from the two-step oxidation of proline to create glutamate stoichiometrically agrees with the quantity of proline used (Sacktor and Childress, 1968). It was expected that the concentration of glutamate would remain relatively constant as it serves as a precursor to α-ketoglutarate via alanine aminotransferase, especially because glutamate is oxidized relatively slowly by mitochondria (Sacktor and Childress, 1968). In the bumblebees, we saw a decrease in glutamate concentration early in flight, which corresponds to the metabolic demands at the onset of flight, when proline is oxidized at higher rates. Glutamate acts as a source for the α-ketoglutarate, and must be transaminated rapidly in order to sufficiently supplement the Krebs cycle and pyruvate oxidation. In the blowfly, this caused an increase in malate concentration at the start of contraction that would

spark the Krebs cycle (Sacktor and Wormser-Shavit, 1966). We did not observe this in bumblebees, and malate remained relatively stable throughout. In the more extensively proline-metabolizing insects like *P. sinuata*, glutamate concentration showed no significant changes throughout flight (Zebe and Gäde, 1993). Once the other amino acids reached steady-state and the initial demand for substrates subsided, we observed an increase and stabilization in glutamate concentration. This may be because carbohydrates act as the primary fuel, and remaining proline oxidation creates glutamate that is not transaminated rapidly because the Krebs cycle does not require priming. Since our results do not parallel those in the blowfly, and knowing that glutamate is oxidized slowly otherwise, there may be a slight build up in concentration. Proline continues to decline at a slow rate, perhaps supplying the oxidative reactions at a rate that steadily produces glutamate as a substrate for AAT.

Evidently, proline is not a primary fuel for flight in *B. impatiens*. In isolated flight muscle tissue, it is oxidized to a greater extent in bumblebees than its hymenopteran counterparts like the honeybee. Barker and Lehner (1972) estimate that only 0.1% of flight energy of worker honeybees is generated through proline, and Micheu et al. (2000) report findings that also suggest honeybees may use some proline during flight. Although the concentration of hemolymph proline in flown bees was significantly lower than in rested bees, the amount metabolized is much lower compared to the use of carbohydrates (Micheu et al., 2000). Crailsheim and Leonhard (1997) suggest that in foragers, the decrease in hemolymph proline seen after returning from flights may be indicative of proline use in foraging metabolism. In the bumblebee, we observed similar relative decreases in the content of trehalose and proline from the maximum to the minimum concentration (approx. 50 and 30%, respectively). Proline appears to contribute more than just marginally to total energy use, given its large decrease. However, proline is not resynthesized like the carbohydrate fuels in order

to sustain flight, so over the course of a longer flight, proline's contribution would be less significant. Furthermore, regulation of proline oxidation might occur at the level of proline dehydrogenase with ADP as the allosteric effector, as demonstrated in the blowfly (Hansford and Sacktor, 1970). It is possible that the requirement of 2 ADP (+2 P_i) for the conversion of glucose to pyruvate reduces the availability of this effector, in turn reducing the rate of proline oxidation as carbohydrate fuel use overrides the need for proline. The contributions of proline oxidation in bumblebees are clearly much greater *in vitro* (Teulier et al., 2016), but *in vivo* it seems the contribution is not appreciably larger than in honeybees. It is also possible that honeybees are not using proline to a larger extent because their flight muscles do not contain sufficient proline dehydrogenase (Crabtree and Newsholme, 1970). It is unclear why the metabolic trait to oxidize proline occurs in bumblebees when they share a diet with insects that rely exclusively on carbohydrates. These findings further emphasize that proline metabolism does not necessitate a protein-rich diet, and using honeybees to represent the metabolic physiology of an order of insects is somewhat flawed (Teulier et al., 2016).

Glycogen to trehalose to glucose – a source for the sink

Trehalose is a disaccharide found in the hemolymph and muscle tissues of many insect species, often acting as the principle blood sugar (Wyatt 1967). Due to its non-reducing power, it can be stored in body fluids at concentrations significantly greater than glucose (Becker et al., 1996). As such, it is available to extensively support insect flight. Trehalose is synthesized in the fat body, a tissue that functions like a liver and fat tissue would in mammals (Becker et al., 1996). Along with triglycerides, the fat body contains glycogen, a large storage molecular composed

primarily of glucose and is the major source of hemolymph trehalose (Arrese and Soulanges, 2011). This trehalose can be homeostatically regulated during exercise-related oxidation by adipokinetic hormones (AKHs) (Gäde and Auerswald, 2002). At the onset of flight, there is a substantial drop in carbohydrate energy substrates because muscles and enzymes are recruited and require the mobilization of stored fuels such as glycogen. The AKH neuropeptides are responsible for mobilizing glycogen through the activation of glycogen phosphorylase (Gäde and Auerswald, 2002). Flight muscles require large amounts of bloodborne trehalose. Since there is no active transport of substrates from hemolymph to tissues, hemolymph trehalose must be maintained at steady and high concentrations in order to supply the flight muscles (Becker et al., 1996).

In many insect species, such as the locust and blowfly, trehalose quantities are reduced after lengthy flights (Bücher and Klingenberg, 1958; Van der Horst et al., 1978a; Sacktor and Wormser-Shavit, 1966). In the African fruit beetle, which uses proline and carbohydrates as co-substrates, there is a decrease in hemolymph trehalose from 13 to 5µmol/ml (Zebe and Gäde, 1993). Since the patterns in bumblebee proline metabolism were similar to what was recorded in the blowfly, we expected to see corresponding patterns in carbohydrate metabolism and replenishment of carbohydrates through glycogen mobilization. Sacktor and Wormser-Shavit (1966) observed trehalose concentration decrease drastically in the blowfly – at a rate of about 1µmol/g of wet weight within the first 5 seconds. This large decrease continued for 30 seconds. For the remainder of the 60-minute flight, the rate of decrease slowed considerably, and there was no plateau in concentration. In our bumblebees, we did not observe the same pattern of trehalose metabolism in the thoraces. Instead of an overall decrease, the trehalose was replenished to quantities seen at rest. However, we did note a large decrease in trehalose concentration in both

flight experiments early in flight. In addition, hemolymph trehalose concentrations remained constant and at higher concentrations than thoracic trehalose.

This pattern in trehalose use suggests that there may be two different sources of trehalose, which behave differently kinetically. Trehalose in the muscle tissues is used up immediately upon initiation of flight, while the trehalose in the hemolymph is depleted slowly during sustained flight (Candy and Kilby, 1975). This also corresponds to the much slower decrease seen during the remainder of flight, and possibly the subsequent increase in thoracic trehalose. It is also possible that there is a homeostatic regulatory mechanism involved in stabilizing or replenishing metabolite levels, so that they either decrease at a slower rate or return to baseline concentrations. This regulation occurs due to the abundant glycogen stores in the insect fat body. Zebe and Gäde (1993) found that glycogen levels in the African fruit beetle remain unchanged for the first 2 minutes of flight, but decrease by more than 50% within 8 minutes in flight muscle. In contrast, they found no changes in the abdomen due to individual variation. Sacktor and Wormser-Shavit (1966) observed glycogen usage in the blowfly during a 60-minute flight. They noted that initially, glycogen in flight muscle did not change. After 2 minutes of flight, glycogen was a major fuel until it was depleted within 10 minutes. In the fat body, they found large depletions occurred even later in flight. In both insects, carbohydrate stores appear to function along a similar timeline, regardless of the extent of proline oxidation. In our bumblebees, a glycogen assay demonstrated that after 30 minutes of flight, abdominal glycogen also decreased to less than half of its concentration at rest. In parallel, abdominal trehalose concentration increased significantly within the same time frame, exceeding the concentration in thoraces by approximately 70%. Our observations suggest that bumblebees are using this abundant sugar to fuel their flight, and maintain concentrations at higher levels in the blood in order to adequately supply the flight

muscles. The glycogen content decreases because it is mobilized in order to produce trehalose that populates both the abdomen and regulates hemolymph concentrations, which remain stable throughout flight. The increase in abdominal content and stable hemolymph trehalose may account for the increase seen in thoracic trehalose at 8 minutes of flight. Overall, a U-shaped curve in thoracic trehalose concentration is seen in both flight experiments, where a sharp decrease in concentration occurs at the onset of flight, and in the second half of the flight, concentrations are replenished to those seen at rest. It is likely that glycogen stores are being mobilized later in flight in order to maintain flight muscle concentrations. This does not occur immediately either, which further emphasizes that proline may be used at the onset of flight due to its ability to supply ATP immediately. Proline use is followed by thoracic trehalose catabolism, which must be sustained by carbohydrate stores.

In order for trehalose to be used in cell metabolism, it must be converted to glucose (Becker et al., 1996). During heightened periods of glycolysis, trehalose cleavage to glucose is promoted (Sacktor and Wormser-Shavit, 1966). Thus, we expected that with the increase in trehalose we observed very early in flight, there would be a corresponding increase in thoracic glucose concentration. In the blowfly, there is a transient increase in glucose concentration at the onset of flight, and concentrations returns to steady state within 30 seconds. In our first flight experiment, it appears as if glucose content is unchanging in the thorax. However, when additional shorter flight times were incorporated, we noticed changes. As trehalose decreases sharply at the onset of flight, we saw a concomitant increase in thoracic glucose by 8 minutes of flight, followed by steady-state concentrations. Hudson (1958) found that the gut delivers large quantities of dietary glucose into the hemolymph during the flight of blowflies, but hemolymph glucose remains relatively constant at a low level. This indicates that there is rapid turnover during flight. Clegg

and Evans (1961) demonstrated that glucose is removed from the hemolymph during flight and is converted into trehalose in the fat body. Furthermore, in the blowfly, hemolymph trehalose is replenished from fat body glycogen and gut sugars. Ultimately, the fat body is the exclusive site for trehalose synthesis (Clegg and Evans, 1961). Kammer and Heinrich (1978) suggest that another reserve of carbohydrates is sugars in the gut, particularly in well fed Hymenoptera and Diptera. As a result, the high glucose gradient across the gut wall facilitates the diffusion of glucose from the gut to the hemolymph. Subsequently, the concentration of glucose is low in the hemolymph because the fat body quickly converts it to trehalose. Although we did detect glucose in the hemolymph of bumblebees, it did not change substantially throughout flight. This may be indicative of rapid glucose turnover, since it is involved in the resynthesis of trehalose in conjunction with glycogen stores.

Abdominal glucose in bumblebees increased largely during flight, possibly to aid in resynthesis of trehalose, while hemolymph glucose remained stable. There are also fewer changes overall in glucose than in trehalose, which suggests high turnover of glucose. Trehalose acts as a source for the glucose sink, being cleaved at rates high enough to ensure that glucose levels remain readily available and stable for cellular metabolism. Lastly, this emphasizes that the overshoots seen in thoracic glucose can reach steady state relatively quickly in an oscillatory fashion, rather than monotonically. This pattern corresponds to the regulatory adjustments seen in the regulation of glycolysis during flight (Sacktor and Wormser-Shavit, 1966). Ultimately, it is clear that during flight, different carbohydrates are mobilized at different rates from different loci in insects, and glycogen is the major vehicle and storage of potential energy. It can be mobilized quickly and with gut glucose, can replenish trehalose in order to meet the metabolic demands of flight muscles. It is also possible to consider that the availability of sugar fuels influences the amount of amino acids

used as metabolic fuels (Brosemer and Veerabhadrappa, 1965). Since bees consume carbohydrates almost exclusively, they likely posses enough carbohydrate reserves to fuel energetically demanding activities. This may be why bumblebees do not continue oxidizing proline as much beyond the initiation of flight – once the Krebs cycle has been primed, carbohydrate fuels can sustain the remainder of the activity.

Supplementation of carbohydrate and Krebs metabolism

If proline acts as a "sparker", we expected proline oxidation to supplement the Krebs cycle, but carbohydrates to still act as the major fuel in bumblebees. It has been demonstrated that the metabolites of the Krebs cycle are regulated similarly in insects that use carbohydrates and those that use proline as major flight fuels (Johnson and Hansford, 1975; Hansford and Johnson, 1976). We expected similar patterns in bumblebees. Flux through the Krebs cycle during insect flight is predominantly controlled by the ADP-activated NAD-isocitrate dehydrogenase reaction (Storey, 1985). The hydrolysis of ATP to ADP at the initiation of flight activates the enzyme and increases Krebs cycle activity. Since proline is oxidized quickly, it can jumpstart this reaction before glycolysis. We detected only AMP and the changes in concentrations did not reflect other findings. AMP increased immensely at the onset of flight in *P. regina*, and decreased to reach a steady state within one minute at a concentration much higher than at rest (Sacktor and Wormser-Shavit, 1966). We observed a decrease in concentration by 30 seconds, but the large increase expected at the onset of flight did not occur. This increase may be absent because the production of ATP through proline oxidation is very fast, so the changes in ATP in insect muscle are minor (Storey, 1985). As a result, changes in AMP may have gone undetected given the flight times we selected.

The patterns observed in bumblebee AMP suggest that both glycolysis and proline oxidation are occurring. Proline enhances pyruvate oxidation in flight muscle tissues *in vitro* when combined with pyruvate and malate, more so than in the absence of proline (Teulier et al., 2016). We expected that there would be large changes in several metabolites due to the demand for ATP. For example, pyruvate is the output of the glycolytic pathway and feeds into the Krebs cycle. It was expected to increase at the start of flight as glucose is used, as well as converted to alanine early in flight (Sacktor and Wormser-Shavit, 1966). Instead, we observed both metabolites predominantly decrease throughout flight. G6P and F6P are precursors to pyruvate in glycolysis. In the first flight experiment, both metabolites slowly decrease until the halfway mark, then increase and reach resting quantities by the end of the flight. In the second experiment, their concentrations remained stable throughout flight, increasing towards the end. This suggests that the high turnover of glucose replenishes the pool of G6P and F6P throughout flight. Decreases seen in trehalose concentration may be keeping these metabolites stable too, since they are downstream of the glucose "sink" at hexokinase. They did not mimic the patterns in the blowfly. The last set of glycolytic intermediates included DHAP and G3P. Both were expected to increase slightly at the start of flight. In both flight experiments, neither of these metabolites changed significantly, and DHAP was present at lower concentrations than G3P, likely because DHAP is reduced to regenerate NAD+ (Kammer & Heinrich, 1978). Theses results align with what we expected in insects that predominantly rely on carbohydrates. Due to the large demand for ATP at the onset of muscle contraction, we observed large changes in glycolytic flux very early in the pathway. The most noticeable changes occurred in glycogen and trehalose, which are energy stores and fuels in glycolysis, respectively. Since these metabolites are used to such a large extent, it is possible that fluctuations further downstream are not occurring at the same magnitude. Sacktor

and Wormser-Shavit (1966) suggest that the glycolytic flux observed on initiation of flight serves as insight into the steps controlling glycolysis *in vivo*: the three sites of regulation include the cleavage of trehalose, phosphorolysis of glycogen, and phosphorylation of F6P. The former two are identifiable in our results.

There is less information about exact quantities of Krebs intermediates in insects during flight. These intermediates are not localized or contained to the Krebs cycle, and can be used in other ways. The Krebs cycle was expected to show a "boost" in intermediates at the onset of flight and achieve steady-state later in flight. Our results corresponded to patterns described generally (Storey, 1985) and in the blowfly (Sacktor and Wormser-Shavit, 1966). Krebs cycle intermediates and their derivatives should be accumulating at the start of flight due to α-glycerophosphate reaction that accounts for over a sixth of total cellular respiration (Chance and Sacktor, 1958). We observed malate at relatively high quantities in both flight experiments, though in the second there is a noticeable decrease by the end of flight. The sharp increase seen in the blowfly does not occur. In contrast, α-ketoglutarate was not expected to change, but we observed a decrease in both thoracic and abdominal concentration. This may occur because this intermediate is required in large quantities for pyruvate oxidation to occur at a rate that sustains ATP production at the initiation of flight. Fumarate behaved similarly to malate in the bumblebee thorax, decreasing towards the end in both flight experiments. Succinate showed a large decrease at the onset of flight. This may be the reason why both malate and fumarate remain relatively stable. Succinate is oxidized rapidly by succinate dehydrogenase in order to produce fumarate, which is converted to malate through fumarase.

Cyclic GMP likely acts as a secondary messenger in the activation of protein kinases through the binding of peptide hormones to cell surfaces. This signaling compound remained

stable throughout both flight experiments. Cyclic AMP is activated by the binding of AKHs to their respective G-protein-coupled receptor (Gäde and Auerswald, 2003), resulting in the activation of tri-acylglycerol lipase, which produces free fatty acids in insects such as the fruit beetle. The role of cGMP was not explored but might be related to a similar process. Hahn and Denlinger (2007) suggest that cGMP-dependent kinases might be involved in feeding behaviour by altering metabolic networks. It might contribute to pre-diapause changes in feeding behaviour, causing reserve accumulation. Although there are differences between species, largely in the Krebs cycle and latter components of glycolysis, many of the fundamental energy use patterns seen in *B. impatiens* correspond to what has been reported in both carbohydrate and proline metabolizing insects. Since the proportions in which the two fuels are used varies between species, we did not expect our findings to perfectly match those of other groups. Flight muscles initially recruit their own metabolic reserves, and contain very small amounts of ATP that do not provide enough energy for even a second of flight (Kammer and Heinrich, 1978; Sacktor and Hurlbut, 1966). Overall, glycolytic intermediates change in quantities that reflect the large role of glycolysis, and the role of proline oxidation becomes clearer as just a substrate to supplement central metabolism.

Relative stability in hemolymph

Hemolymph is analogous to blood and circulates throughout the arthropod body, maintaining contact with tissues. It is extensively studied in insect orders like Coleoptera, Diptera, and Hymenoptera, and is known to be rich in compounds including carbohydrates and sugars, proteins, lipids, amino acids, ammonia, and urea (Wyatt, 1961). We detected amino acids, carbohydrates, sugars, and intermediates of the Krebs cycle and glycolysis in the hemolymph of

B. impatiens. In honeybees, hemolymph contains high concentrations of free amino acids. Not much is known about their role, though they may be involved in osmoregulation. Crailsheim and Leonhard (1997) found that glutamic acid and alanine were the predominant amino acids in hydrolysates, and proline was the most abundant free amino acid, reaching its peak concentration in 3-day old honeybees at 25.8nmol/µl, or 80% of amino acid content. We detected approximately 4µmol/mL in our resting bumblebee hemolymph, which is still lower than the 15nmol/µl in the older honeybees from the same study. In dipterans like houseflies, for example, hemolymph proline concentration can reach up to 78.3mg/100mL (Price, 1961). The amounts of sugars in hemolymph also vary greatly, so it was only expected that our findings would fall within the range of values reported in literature. The concentration of trehalose, for example, should exceed the concentration in the thorax by a factor of 5 to 15 (Sacktor and Wormser-Shavit, 1966), which we noted in our results. Trehalose concentration in hemolymph ranges from 2mg/mL (Bounias and Morgan, 1984) to 40mg/mL (Bozic and Woodring, 1997). Glucose and fructose were found to range from 2mg/mL (Abou-Seif et al., 1993) to 15mg/mL (Fell 1990; Leta et al., 1996). Abou-Seif et al. (1993) also found that the decrease in trehalose during flight was accompanied by increases in glucose and fructose. Fell (1990) noted that although mean hemolymph sugar concentrations in literature were similar, individual variability is very high, likely due to metabolic differences. Lastly, Leta et al. (1996) observed hemolymph sugars in bees preparing to swarm, and confirmed that the predominant hemolymph sugars in adult worker honeybees were trehalose, glucose, and fructose. Our results show that these sugars are present in hemolymph, though below the lowest reported concentrations, possibly due to error. Since the quantities we detected are so low, we can also assume that the metabolites in the blood were exported by the cell. Tissue damage can occur *in vivo* during exercise, causing metabolites and enzymes to leak out and ultimately skewing

concentrations that would be naturally found in the hemolymph, though this does not appear to be the case in our bees. Homeostatic regulation tightly controls hemolymph carbohydrate levels, resulting in relatively constant levels of hemolymph sugars despite differently behaving groups of bees (Leta et al., 1996). This can be linked to hormonal regulation of honeybee blood sugars by insulin- and glucagon-like peptides (Maier et al., 1988, 1990).

Ultimately, we did not observe the same decreases in bumblebee hemolymph metabolites that have been documented in other species. Despite changes seen in tissue and abdominal metabolites, none of the hemolymph metabolites changed throughout the flight experiments, with the exception of fumarate. We expected that trehalose and proline would decrease due to their roles as primary fuels in many species, whereas glucose might increase at the onset of flight, but otherwise remain stable. Our results do not adhere to patterns in literature, other than the concentration of trehalose in the hemolymph largely exceeding that in the thorax. In terms of amino acids, although concentrations of proline are high, the hemolymph contains considerably less than what is found in the thorax (Price, 1961; Sacktor and Wormser-Shavit, 1966). Crailsheim and Leonhard (1997) found lower values of free amino acids in foraging bees, but individual amino acids showed higher variability of concentration than overall amino acid content, so it was unclear why the decrease had occurred. They also found that while all other amino acids declined during flight, proline concentration remained constant. It was strange to not observe a decrease in proline in bumblebees, which oxidize it to a greater extent *in vitro* than honeybees. Possible physiological reasons include tight homeostatic regulation of metabolic pathways, and high turnover of hemolymph metabolites. We know flux is still occurring due to the law of mass action, and products may remain at more constant concentrations because the reactant is able to supply them, leaving the metabolites in a state of equilibrium. Due to the lack of change between time points, it

is difficult to make conclusions about metabolites' involvement in bumblebee flight, especially those that are considered fuels. Additional reasons for this outcome might include high variability of free amino acids, different groups of bees, nutritional or seasonal differences, and high standard error contributing to lack of observable change (Crailsheim and Leonhard 1997). Alternatively, technical reasons for such low detection could be that the injected hemolymph volume was too low and diluted for optimal metabolite detection.

Overall change in the metabolome

Principal component analysis (PCA) allows us to observe changes in multiple variables simultaneously, and see strong patterns in large datasets. The PCA we performed allows us to assess overall changes in metabolic profiles of individual bees. For example, for one bee a metabolite may not change much but in another it may dominate, and PCA allows us to see patterns combining several connected variables. Changes seen in metabolic profiles at each time point contribute to how the clusters shift in the scores plot, and significant shifts are more evident when graphs are generated from the mean PC scores over the course of flight (Fig. 1 and 2, C., D.). The biplots allow us to identify whether metabolites share a response profile, and the magnitude of the effect of flight time, in terms of the variation displayed by the metabolite for that dataset. When the biplots and scores plots generated for the thoracic datasets are observed alongside the bar graphs for changes in individual metabolites, we see the response profiles of many metabolites in biplots correspond to what is seen in bar graphs.

The overarching patterns in biplots was that the response profiles of carbohydrate fuels and intermediates are opposite to proline. The length of the vectors for these metabolites exceeds

others, and dominates PC1. It is clear that the effect of flight on proline and trehalose is greatest, suggesting that although they are used differently, they are both used as fuels to a large extent and concomitantly show the most variation in the dataset. Since the magnitude of both effects appears similar, it is difficult to discern to what extent they are used as fuels. A second finding is that many glycolytic and Krebs intermediates do not show the same response profile in both experiments. Metabolites from these categories, as well as alanine, dominated PC2 in both flight experiments. The magnitude of their responses is much smaller, demonstrated by vector size. This is likely because these intermediates are further downstream, and not necessarily localized to be involved in the Krebs cycle alone. We also see that alanine does not show an opposite response to proline. This suggests there is no equimolar relationship between the two amino acids, and proline is not being resynthesized. Given its response, it is probably being used as a fuel, only to supplement the Krebs cycle.

The scores plots show shifting clusters that represent the metabolite profiles of individual bees. The clusters reflect changes occurring at different time points. Overall, we see that there are several large shifts occurring in both flight experiments, predominantly during the onset of flight (0 to 2 minutes) and at the end of flight (15 to 30 minutes). We also saw that at these periods there was a lot more variation between individual metabolite profiles, and the clusters span across PC1 or 2. In contrast, in both experiments we observed less variation between individual profiles at 8 and 15 minutes of flight, and these profiles tended to overlap as well. This suggests that perhaps metabolites have actually achieved steady state at this point, and there is overall less deviation from this trend. These patterns reflect the model of flight metabolism proposed by Zebe and Gäde (1993), especially because after stabilization, metabolites often return close to concentrations seen at rest, or exhibit a similar variation. Ultimately, biplots allow us to better understand which

metabolites act together. Scores plots are more exploratory and showed variation between individuals and how the metabolome changed overall throughout flight. These analyses do not describe exactly what happens at each time point, but in conjunction with bar graphs generated for individual metabolites, patterns in their fluctuations become more evident and correspond to trends in literature.

Alternative uses for proline

Despite the changes occurring in thoracic proline content, it is possible that this amino acid is not being used to fuel flight. Although *B. impatiens* is able to oxidize proline *in vitro* and appears to use this amino acid at the onset of flight as a sparker, many other related species do not possess this ability. It is not certain why this trait evolved in this species. In the case that it is not used for flight, this phenotype may be important during periods when dietary sources of carbohydrates are scarce, like in early spring or overwintering (Teulier et al., 2016). The fat body is essential for energy storage in insects, and it also synthesizes many of the circulating metabolites and hemolymph proteins (Arrese and Soulages, 2010), including proline through AKHs (Gäde & Auerswald, 2002). Proline oxidation may be involved with mobilizing the fuels stored in the fat body. This includes fatty acids that cannot be oxidized directly (Arrese and Soulages, 2010), but proline can act as a shuttle for the carbon (Gäde & Auerswald, 2002; Bursell 1977).

Proline oxidation is a potentially advantageous trait otherwise. Gradually increasing temperatures can impact the diapause of northern, cold-adapted bumblebee species (Versterlund et al., 2014). Insects that overwinter at warmer temperatures have increased respiration and show greater consumption of their energy reserves than insects that overwinter at lower temperatures

(Irwin and Lee, 2000; Thompson and Davis, 1981). This could lead to harmful weight loss in bee species due to loss of energy stores (Fründ et al., 2013). However, the partial oxidation of proline results in 0.52 mol of ATP per gram of this fuel, which makes it more similar to lipids (0.65 mol ATP) than carbohydrates (0.18 mol ATP) in terms of energy production (Gäde & Auerswald, 2002; Bursell 1981). Furthermore, alanine produced during partial oxidation can be resynthesized to proline, more quickly at rest. It is possible that when carbohydrate fuels are scarce, the proline found in the limited quantity of plant nectars acts as a more robust source of energy for Hymenoptera with this metabolic trait.

The emergence of entometabolomics and sources of variation

Metabolomics experiments help understand biochemical responses in cells and organisms that can be caused by physiological demands (Gil et al., 2015). In this study, a targeted rather than global approach was chosen in order to understand how specific metabolic pathways and their intermediates are affected by flight, an energetically demanding activity. It was possible to detect many of the metabolites and intermediates initially targeted, which allowed us to create snapshots of the functional metabolic phenotype at various points throughout the flight. UPLC also provides the largest known liquid chromatographic resolution and peak capacity. Coupled with sensitive mass spectrometers, it is possible to separate and identify metabolites within complex mixtures, like biofluids (Forcisi et al., 2013). Ultimately, similar patterns between the two thoracic datasets were found among many metabolites, corresponding to patterns in literature.

Despite the advantages of metabolomics, it is important to note that results obtained through these approaches are not fully reproducible. Although many variables were controlled to

the best of our abilities, differences between datasets arose. The chemical nature of the metabolome is so diverse that there is no single method that can capture all the metabolites, even within a targeted search (Barnes et al., 2016). It was possible to create metabolic profiles of bumblebees that correspond to findings in literature, and ultimately if all the targeted metabolites were detected, it would be possible to generate substrate to product ratios with pairs of metabolites. This would allow us to understand changes in enzyme activity – it would behave much like a dam, where the decrease in one metabolite is seen as an increase in the subsequent intermediate. However, there were difficulties in detecting components of oxidative phosphorylation and the Krebs cycle. This creates several gaps in the pathways, though it is possible to hypothesize what might occur by supplementing with additional assays. Our inability to detect certain metabolites with our method does not necessarily mean they are not present in the biological system. They are present because they are required in cellular metabolism, though many metabolites (like ATP and ADP) are also subject to chemical degradation and may interconvert to other metabolites of interest (Gil et al., 2015), or are incompatible with the solvent system we used. Compounds belonging to more sensitive classes require adjustments in pH, using additives and working with other solvent combinations, or employing techniques like NMR.

Furthermore, metabolites comprise a very downstream "-ome," so changes in the metabolome are amplified a lot more than what occurs in the transcriptome or proteome (Gil et al., 2015). Diet is another factor that can contribute to unreproducible results. All insects should be on controlled diets that come from a single batch in order to avoid variability in diet causing biological effects (Barnes et al., 2016). Our bees were not from a single colony (albeit from the same source), and were able to feed freely, so there was no control over how much individuals consumed. It is possible that diet is what contributed to the large differences in some metabolites between the two

tissue experiments. Pollen might account for the 2µmol difference seen in thoracic proline concentrations at rest, and sucrose solution might influence disparities between carbohydrate and glycolytic metabolites. Additionally, age largely affects metabolite quantities in bees. Crailsheim and Leonhard (1997) found that overall amino acid concentration peaks in 3-5 day old honeybees, and decreases in older bees. Leta et al. (1996) noted that glycogen levels in the abdomen and thorax increases with age as well. Lastly, it is difficult to compare flight experiments because these methods are not standardized. For example, Scaraffia and Wells (2003) conducted flight experiments with mosquitos in order to observe the activity of proline as a substrate during flight. The time required to collect hemolymph from insects post-flight, especially in shorter flight times, gave rise to inconsistent results. Similarly, our hemolymph results tended to show higher standard error and unclear patterns in metabolite changes, presumably due to small volumes and the time required to collect hemolymph.

Conclusion

The flight experiments conducted on *B. impatiens* and generation of metabolic profiles revealed patterns in fuel use that support the role of proline as a "sparker". For example, proline decreases largely at the onset of flight and there is no concomitant increase in alanine. Carbohydrate fuels are used almost immediately in order to generate ATP for sustained flight. There is an overall decrease in glycogen content in the abdomen, supplementing trehalose in the flight muscles. Trehalose shows some decrease largely at the start of flight but remains stable for the remainder, suggesting the involvement of a homeostatic regulatory mechanism in the glycogen-trehalose-glucose axis. Remaining metabolites behave similarly to what has been

previously observed in the blowfly, particularly the Krebs cycle intermediates. It can be concluded that carbohydrates are the major energy substrate, while proline acts to supplement Krebs cycle intermediates largely at the start of flight. Although it is difficult to recreate flight experiments due to the lack of standardization and variation between metabolomic datasets, the patterns observed in our findings support the use of proline as a supplementary fuel. There may also be alternative reasons for why the metabolic phenotype to oxidize proline to this extent evolved in bumblebees. It is not present in all hymenopterans, so further insight is required to understand its role in overwintering, or if it is the outcome of a dietary adaptation or preference in bumblebees.

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