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RESEARCH ARTICLE

Overwintering in North American domesticated honeybees (*Apis mellifera*) causes mitochondrial reprogramming while enhancing cellular immunity

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ABSTRACT

Many factors negatively affect domesticated honeybee (*Apis mellifera*) health, causing a global decrease in their population year after year with major losses occurring during winter, and the cause remains unknown. Here, we monitored for 12 months North American colonies of honeybees enduring important temperature variations throughout the year, to assess the metabolism and immune system of summer and winter honeybee individuals. Our results show that in flight muscle, mitochondrial respiration via complex I during winter is drastically reduced compared with summer. However, the capacity for succinate and glycerol-3-phosphate (G3P) oxidation by mitochondria is increased during winter, resulting in higher mitochondrial oxygen consumption when complex I substrates, succinate and G3P were assessed altogether. Pyruvate kinase, lactate dehydrogenase, aspartate aminotransferase, citrate synthase and malate dehydrogenase tend to have reduced activity levels in winter, unlike hexokinase, NADH dehydrogenase and pyruvate dehydrogenase. Transcript abundance of highly important immunity proteins such as *Vitellogenin* and *Defensin-1* were also increased in winter bees, and a stronger phagocytic response as well as a better hemocyte viability was observed during winter. Thus, a reorganization of substrate utilization favoring succinate and G3P while negatively affecting complex I of the ETS is occurring during winter. We suggest that this might be due to complex I transitioning to a dormant conformation through post-translational modification. Winter bees also have an increased response for antibacterial elimination. Overall, this study highlights previously unknown cellular mechanisms between summer and winter honeybees that further our knowledge about this important species.

KEY WORDS: Metabolism, Seasonal transition, Immune system, Phagocytosis, Substrate oxidation

INTRODUCTION

The western honeybee (*Apis mellifera*) can be found nearly worldwide and plays a substantial role in ecosystems as one of the most economically valuable pollinators (Esquivel et al., 2021; Winfree et al., 2011). The population decline of honeybees owing to a combination of different threats in recent years has raised concerns for their global health worldwide both at the scientific and at the public levels (Potts et al., 2010). One such threat is the occurrence of extreme weather events and the increased temperature variations brought forth by climate change (Vasseur et al., 2014). For example, in the last 15 years, there have been dramatic honeybee winter colony losses being frequently reported from different regions all over the world (Currie et al., 2010; Gray et al., 2020; Potts et al., 2016; Steinhauer et al., 2021). This climate variability (and notably at the level of temperature variations) has been shown to be one of the driving factors contributing to the honeybee population losses (Calovi et al., 2021; Switanek et al., 2017). It is thus of paramount importance to investigate the physiology of honeybees subjected to environmental conditions throughout the year to know whether and how these insects will adapt and survive in the future.

Honeybees are ectotherms depending on the environmental temperature, and can live in colonies, considered as superorganisms (Canciani et al., 2019). As a colony, they have developed behavioral endothermic strategies to regulate the temperature inside the hive during both cold and warm temperatures (Stabentheiner et al., 2012; Stupski and Schilder, 2021). Hence, the physiology of honeybees changes drastically according to the different seasons. Specifically, winter and summer honeybees have contrasting phenotypes. The more drastic seasonal difference is their lifespan: summer and spring worker bees have an average lifespan of 25–40 days compared with winter bees, which can live between 212 and 252 days (Mattila et al., 2001). Long-lived winter honeybees, known as diutinus bees, have also enlarged fat bodies compared with summer honeybees owing to the important accumulation of nutrients for winter (Fluri and Bogdanov, 2015; Knoll et al., 2020). Diutinus bees are also known to have higher levels of hemolymph proteins, predominantly vitellogenin (Vg), a protein that regulates immune function and is associated with longevity in queens and winter honeybees and therefore is considered of great importance for overwintering (Amdam et al., 2003; Döke et al., 2015; Kunc et al., 2019; van der Steen et al., 2015). Vg is also involved in phagocytosis, one of the most important cellular immune responses in winter bees and forager bees (Gätschenberger et al., 2013; Hystad et al., 2017; Salmela et al., 2015). Specifically, honeybee Vg binds to surface molecules of pathogens (i.e. lipopolysaccharides), resulting in the activation of the phagocytotic process in hemocytes (Hystad et al., 2017; Salmela et al., 2015). Immune gene transcript abundance has been demonstrated to significantly change in overwintering bees compared with summer bees. For example, *Vg* mRNA levels are known to be higher in winter than in summer bees, as well as the mRNA level of *defensin-1* (*Def1*), an antimicrobial peptide (AMP) important for social immunity (Dostálková et al., 2021). Winter honeybee populations also show greater potential to induce immune responses than summer populations after immune stimuli (Dostálková et al., 2021). However, it is important to consider that any immune reaction is associated with high energetic costs

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and that the immune system becomes privileged in terms of using organismal resources during a pathogen infection (Dolezal et al., 2019; Gätschenberger et al., 2013). Specifically, a systemic metabolic switch redirecting nutrient flow towards immunity and away from consumption by non-immune process occurs during an immune response to enable the rapid production of ATP and new biomolecules (Dolezal et al., 2019). In that regard, the insect fat body plays a dual role as it serves as both a metabolic organ, storing energy reserves and providing fuels to the rest of the organism, and an important organ for humoral immunity (Dolezal et al., 2019; Li et al., 2019). For example, the fat body synthesizes trehalose (an insect specific carbohydrate composed of two glucose molecules), which is transported to skeletal muscles to power flight, but also AMPs and vitellogenin, which are crucial for immunity (Bretscher and O'Connor, 2020; Dolezal et al., 2019; Li et al., 2019; Suarez et al., 2005). Thus, the regulation of metabolism during an immune response is crucial for the survival of organisms because the energy/nutrient needs of the immune system might interfere with the needs of other physiological traits such as muscle contraction for foraging and shivering.

In the hive, several castes co-exist during summer, such as drone, queen and worker. For the worker caste, newly emerged bees take on several roles in the hive depending on their age (cell cleaner, nurse, comb building, colony maintenance, nectar processing) until they become forager bees ∼21 days after emerging from the cell of the honeycomb (Ament et al., 2010; Robinson, 2003). Once becoming a forager bee, they have the role of retrieving pollen and nectar from plants and bringing them back to the hive, which is energetically demanding. In comparison, during the winter season, worker bees do not leave the hive and form a cluster that surrounds the queen to keep her warm. When ambient temperatures drop below ∼15°C, the honeybee colony starts to form the cluster, maintaining a steady-state temperature of ∼35°C in the colony's central brood area where the queen is located (Southwick and Heldmaier, 1987; Stabentheiner et al., 2003). The colder the ambient temperature becomes, the tighter the cluster will be to prevent heat loss by reducing the effective area of heat exchange, which is a decisive factor for survival at low temperatures (Southwick, 1983; Southwick and Heldmaier, 1987; Stabentheiner et al., 2003). In the inner part of the cluster, the heater bees are the ones who perform 'endothermic heating' by thorax muscle contractions (shivering). Another group of bees fan their wings to dissipate the heat and regulate the CO_2 levels (Southwick and Heldmaier, 1987; Stabentheiner et al., 2003; van Nerum and Buelens, 1997). In some parts of the world, winter temperatures can go down well below 0°C for several days, which can make it very difficult for honeybee clusters to keep warm inside the hive. During these stressful periods, honeybees can raise their metabolic rate by up to 25 times to produce the necessary heat (Southwick and Heldmaier, 1987). This energetically demanding mechanism requires a constant supply of nutrients, mainly carbohydrates that can be found in the honey within the hive that is used as metabolic fuel throughout winter. Although no proper comparisons of metabolic rates have been performed between summer and winter honeybees, it has been shown that individual forager bees during summer increase their metabolic rate (measured at 30°C) by ∼15.5 times during flight (Harrison and Fewell, 2002; Harrison and Roberts, 2000), whereas in honeybees in clusters, metabolic rate can increase by up to 25 times at external temperatures below 10°C (Southwick and Heldmaier, 1987).

Regardless of the ambient temperature, the in-hive climate of a beehive at the central brood must be kept at the average optimum temperature of 32–36°C as homeostatic set point for the colony's survival (Ocko and Mahadevan, 2014; Southwick and Heldmaier, 1987). Considering the important energetic cost for the maintenance of such temperature during winter, physiological and metabolic functions should display high divergence between summer and winter individuals, and the control of several metabolic pathways should change accordingly to reflect this divergence. This should be particularly important for the aerobic metabolism and mitochondrial oxidative phosphorylation that provides the vast majority of ATP for the cells. Mitochondria are the metabolic hub of organisms, and in the case of honeybees, they must properly balance energy supply and demand to fine-tune the metabolic needs of the hive (thermogenesis in winter and foraging in summer) throughout the year according to the contrasting temperature variations. However, it is surprising that mitochondrial functions have not yet been examined in individuals displaying these summer and winter contrasting phenotypes.

The aim of this study was to investigate whether and how honeybee metabolism and immune system are modulated during seasonal transitions. For the evaluation of metabolism, we used thorax muscles, as they are used in shivering during winter and flying during summer, which are both energetically costly processes, and are very dense in mitochondria. Specifically, we evaluated mitochondrial respiration at several steps of the electron transport system (ETS), enzymatic activities of several metabolic pathways, and transcript abundance of genes encoding mitochondrial subunits of the ETS, as well as transcript abundance of *Vg* and *Def1* in these muscles, and determined the phagocytic capacity and viability of hemocytes in the hemolymph to obtain a cohesive picture of the immune and metabolic changes occurring throughout the year. We hypothesized that metabolic parameters such as mitochondrial respiration, activity of aerobic enzymes and transcript abundance of mitochondrial genes of winter individuals will be globally increased in comparison to summer bees to maintain temperature homeostasis by sustaining the shivering thermogenesis required for the survival of the colony. Moreover, metabolic needs and immunity in insects are mechanistically linked to the dual role of the fat body to provide nutrients to skeletal muscles and be responsible for the humoral immune response. Considering that external infections of the bee cluster are unlikely during winter (Gätschenberger et al., 2013) and that any immune reaction is coupled with high energetic costs, we hypothesized that thermogenesis will be privileged in terms of metabolic needs by redirecting nutrients to fuel this process over the immune system, and that immune functions determined by hemocyte immune capacity and transcript abundance of *Vg* and *Def1* will weaken as a result.

MATERIALS AND METHODS

Experimental model

Three smart experimental hives (Amohive®, Poland) recording real-time temperature, humidity and mass every 20 min (see Fig. S1 for a complete temperature profile) were put on the rooftop of the Université de Moncton (Moncton, NB, Canada, 46°6′25.92″N, 64° 47′18.24″W). The hives consisted of polystyrene boxes 60.64 cm wide by 50.48 cm long with a depth of 24.45 cm. The boxes were stacked (two during winter and autumn and three during summer and spring) and every box included 10 waxed brood wood frames allowing optimal hive construction by honeybees.

For a period of 1 year, honeybees (*Apis mellifera* Linnaeus 1758) were sampled from the three colonies. Every sampling took place consistently each month (±10 days) between September 2020 and August 2021. Summer and spring bees (foragers) were sampled

exiting or entering the entrance of the hive. During autumn and winter, we sampled bees inside the hives on top of the frames located at the periphery of the clusters. During winter 2021, precisely in February, two out of the three colonies died, thus sampling was only performed from the remaining hive. The collected bees were brought back to the laboratory (1 km from the sampling site) and were anesthetized on ice for approximately 10–15 min before the dissection of the thorax muscle or hemolymph extraction for mitochondrial respiration and flux cytometry, respectively. Honeybees were also stored at −80°C for further enzymatic activity measurements and molecular biology experiments. To reduce *Varroa destructor* infestation levels and ensure overwintering survival, hives were treated with formic acid in August/September and with oxalic acid in December.

Mitochondrial respiration experiments

Tissue sampling and permeabilization

Permeabilization and dissection of the fibres were conducted on ice, according to Menail et al. (2022) and Simard et al. (2018). Specifically, after dissection of the thorax, the collected muscles were rapidly placed in 1 ml of ice-cold relaxing solution BIOPS (2.77 mmol l^{-1} CaK_2EGTA, 7.23 mmol l^{-1} K_2EGTA, 5.77 mmol l^{-1} Na_2ATP 6.5 mmol l^{-1} $MgCl_2$, 20 mmol l^{-1} taurine, 15 mmol l^{-1} Na_2Phosphocreatine, 20 mmol l^{-1} imidazole, 0.5 mmol l^{-1} dithiothreitol and 50 mmol l^{-1} K-MES, pH 7.1) for mechanical permeabilization (Pesta and Gnaiger, 2012). The muscle fibers were then transferred to BIOPS complemented with 62.5 µg ml^{-1} of saponin and placed on an orbital shaker for 15 min at 220 rpm. The muscle fibers were then transferred in 1 ml of respiration medium [120 mmol l^{-1} KCl, 5 mmol l^{-1} KH_2PO_4, 3 mmol l^{-1} Hepes, 1 mmol l^{-1} EGTA, 1 mmol l^{-1} $MgCl_2$ and 0.2% bovine serum albumin (w/v), pH 7.2 (Pichaud et al., 2010)] on a shaker for 5 min at 220 rpm. Muscle fibers were then put on drying paper to absorb excess buffer and weighed on a Secura 225D-1s semi-microbalance (0.01 mg readability, Sartorius, Göttingen, Germany) for acquisition of 0.5–1 mg of tissue.

Measurement of mitochondrial respiration rates

Rates of mitochondrial oxygen consumption (N=8–12) were measured with a high-resolution respirometer (Oxygraph-O2K, OROBOROS Instruments, Innsbruck, Austria). All measurements were performed at 35°C (Stabentheiner et al., 2010; Teulier et al., 2016) with air-saturated respiration medium. All experiments were performed at concentrations between 50 and 200 µmol l^{-1} of oxygen in the respirometer and re-oxygenation was performed if the oxygen was below 50 µmol l^{-1}. Permeabilized thorax muscles were transferred into each chamber of the respirometer filled with air-saturated respiration medium with pyruvate (10 mmol l^{-1}), malate (2 mmol l^{-1}) and glutamate (10 mmol l^{-1}). The signal was allowed to stabilize to measure the LEAK respiration at the level of complex I (CI-LEAK).

The following substrates, uncoupler and inhibitors were then added sequentially to the chambers as previously described (Cormier et al., 2019; Menail et al., 2022; Simard et al., 2018). First, injection of ADP (5 mmol l^{-1}) enabled the measurement of mitochondrial oxygen consumption when the transport of electrons from complex I is coupled to the phosphorylation of ADP to ATP (CI-OXPHOS). The functional integrity of the outer membrane was then verified by adding 10 µmol l^{-1} of cytochrome *c* (CIc-OXPHOS; Kuznetsov et al., 2008). Proline (5 mmol l^{-1}), a substrate known to have great importance for mitochondrial respiration in some flying insects (Teulier et al., 2016) and provides electrons to the ETS via the proline dehydrogenase (CI+ProDH-OXPHOS), was then added. This was followed by succinate (20 mmol l^{-1}), which brings electrons to complex II (CI+ProDH+CII-OXPHOS), and G3P (15 mmol l^{-1}), which allows the transport of electrons to the ETS via the mtG3PDH (CI+ProDH+CII+mtG3PDH-OXPHOS). These different substrates were used to evaluate their relative contribution to mitochondrial oxygen consumption (see below). Complexes I, II and III where then inhibited by rotenone (0.5 µmol l^{-1}), malonate (5 mmol l^{-1}) and antimycin A (2.5 µmol l^{-1}), respectively, to evaluate the residual oxygen consumption, which was subtracted from the previous respiration rates measured. Finally, ascorbate (2 mmol l^{-1}) and *N*,*N*,*N*′,*N*,-tetramethyl-*p*-phenylenediamine (TMPD, 0.5 mmol l^{-1}) were added to evaluate the maximum capacity of complex IV (CIV), which was corrected for auto-oxidation of TMPD after inhibition of complex IV by sodium azide (20 mmol l^{-1}). Respiration rates are expressed as pmol O_2 s^{-1} mg^{-1} tissue.

Calculation of mitochondrial ratios

The respiration rates measured were used to calculate different ratios (N=8–12). First, we calculated the coupling ratio at the level of complex I (1–CI-LEAK/CI-OXPHOS). The cytochrome *c* effect (CIc-OXPHOS/CI-OXPHOS) was then calculated to verify the integrity of the outer mitochondrial membrane: if an increase of more than 10% was observed after the injection of cytochrome *c*, the preparation was discarded (Kuznetsov et al., 2008). Moreover, we also calculated the contributions of each substrate to mitochondrial respiration, i.e. proline contribution=(CI+ProDH-OXPHOS−CIc-OXPHOS)/CIc-OXPHOS, succinate contribution=(CI+ProDH+CII-OXPHOS−CI+ProDH-OXPHOS)/CI+ProDH-OXPHOS, and G3P contribution=(CI+ProDH+CII+mtG3PDH-OXPHOS−CI+-ProDH+CII-OXPHOS)/CI+ProDH+CII-OXPHOS).

Determination of enzymatic activity

For enzymatic activities, the thoraxes were separated from the head and the abdomen. Two thoraxes were weighed and homogenized in a potassium phosphate buffer (PBS), containing 6.1 mmol l^{-1} of K_2HPO_4 and 39 mmol l^{-1} of KH_2PO_4, pH 7.0. Samples were then centrifuged at 800 ***g*** for 5 min at 4°C, and the supernatant was stored at −80°C until measurement of enzymatic activities. Enzymatic activities were determined with a BioTek Synergy H1 microplate reader (Biotek, Montréal, QC, Canada). Assays were performed at 35°C (N=6–8). Protocols for enzyme activities were modified from Bergmeyer et al. (1983) and Ekström et al. (2017). All enzymatic activities are expressed as mU mg^{-1} tissue, where U represents 1 µmol of substrate transformed to product in 1 min.

Hexokinase (HK, EC 2.7.1.1) activity was determined by following the production of NADPH at 340 nm (ε=6.22 ml cm^{-1} $µmol^{-1}$) for 8 min. The reaction medium contained 222 mmol l^{-1} glucose, 8 mmol l^{-1} $MgCl_2$, 0.64 mmol l^{-1} ATP, 0.91 mmol l^{-1} NADP and 0.55 U ml^{-1} glucose-6-phosphate dehydrogenase in 100 mmol l^{-1} PBS, pH 7.0.

Pyruvate kinase (PK, EC 2.7.1.40) activity was measured by recording the disappearance of NADH at 340 nm (ε=6.22 ml cm^{-1} $µmol^{-1}$) in a reaction medium containing 10 mmol l^{-1} $MgCl_2$, 100 mmol l^{-1} KCl, 5 mmol l^{-1} ADP, 0.15 mmol l^{-1} NADH, 5 mmol l^{-1} phosphoenolpyruvate and 0.08 U ml^{-1} lactate dehydrogenase in 50 mmol l^{-1} imidazole-HCl buffer, pH 7.4 for 8 min.

Lactate dehydrogenase (LDH, EC 1.1.1.27) activity was measured by following the disappearance of NADH at 340 nm (ε=6.22 ml cm^{-1} $µmol^{-1}$) for 8 min. The reaction medium

contained 0.16 mmol l^{-1} NADH and 0.4 mmol l^{-1} pyruvate in 100 mmol l^{-1} PBS, pH 7.0.

Pyruvate dehydrogenase (PDH, EC 1.2.4.1) activity was measured at 490 nm following the reduction of idonitrotetrazolium (INT) (ε=15.9 ml cm^{-1} μmol^{-1}) for 8 min. The reaction medium contained 2.5 mmol l^{-1} NAD, 0.5 mmol l^{-1} EDTA, 0.1 mmol l^{-1} coenzyme A, 0.1 mmol l^{-1} oxalate, 0.6 mmol l^{-1} INT, 0.5 U mL^{-1} diaphorase, 0.2 mmol l^{-1} thiamine pyrophosphate and 5 mmol l^{-1} pyruvate in Tris-HCl buffer (50 mmol l^{-1} Tris base, 0.1% v/v Triton X-100, 1 mmol l^{-1} $MgCl_2$, 1 mg ml^{-1} BSA), pH 7.8.

Citrate synthase (CS, EC 2.3.3.16) activity was determined by following the reduction of 5,5′-dithiobis (2-nitrobenzoic acid) (DTNB) (ε=14.15 ml cm^{-1} μmol^{-1}) at 412 nm for 8 min. The reaction medium contained 0.1 mmol l^{-1} DTNB, 0.1 mmol l^{-1} acetyl-CoA and 0.13 mmol l^{-1} and the reaction was started with the addition of oxaloacetic acid in 100 mmol l^{-1} imidazole-HCl, pH 7.4.

Malate dehydrogenase (MDH, EC 1.1.1.37) activity was measured by following the disappearance of NADH at 340 nm (ε=6.22 ml cm^{-1} μmol^{-1}) for 8 min in a reaction medium containing 0.2 mmol l^{-1} NADH and 0.5 mmol l^{-1} oxaloacetic acid in 100 mmol l^{-1} PBS, pH 7.0.

Aspartate aminotransferase (AAT, EC 2.6.1.1) activity was measured by following the disappearance of NADH at 340 nm (ε=6.22 ml cm^{-1} μmol^{-1}) for 8 min in a reaction medium containing 0.025 mmol l^{-1} pyridoxal phosphate, 0.32 mmol l^{-1} NADH, 10 mmol l^{-1} alpha-ketoglutarate, 22 mmol l^{-1} aspartate and 0.6 U ml^{-1} malate dehydrogenase, in PBS, pH 7.0.

NADH dehydrogenase (CI, EC 7.1.1.2) activity was measured at 600 nm for 10 min by following the reduction of 2,6-dichloroindophenol sodium salt hydrate (DCPIP) (ε=19.1 ml cm^{-1} μmol^{-1}). The reaction medium contained 0.5 mmol l^{-1} EDTA, 3 mg ml^{-1} BSA, 1 mmol l^{-1} $MgCl_2$, 2 mmol l^{-1} KCN, 4 µmol l^{-1} antimycin A, 75 µmol l^{-1} DCPIP and 65 µmol l^{-1} coenzyme Q1 in 100 mmol l^{-1} PBS, pH 7.0. The samples were incubated with the reaction medium for 1 min before adding NADH (0.14 mmol l^{-1}) to start the reaction. The same reaction was measured with the addition of rotenone (10 µmol l^{-1}) to determine rotenone-sensitive NADH dehydrogenase activity.

RNA extraction and transcript abundance

Honeybees stored at −80°C were dissected on dry ice and only the thorax was used for the extraction (*N*=5–8). Total RNA was extracted using TRIzol reagent (Sigma-Aldrich, St Louis, MO, USA) according to the manufacturer's protocol. The 260 nm/280 nm absorbance ratio was used to verify the quality of the RNA in each sample. Total RNA (1 µg) was reverse transcribed using the Bioline SensiFAST cDNA Synthesis Kit (Bioline, London, ON, Canada). Real-time quantitative PCR was performed on a CFX Connect (Bio-Rad Laboratories, Hercules, CA, USA) by incubating the cDNA with forward and reverse primers (0.42 µmol l^{-1}) and by using the 2× SensiFAST™ SYBR No-ROX kit (Bioline, London, ON, Canada) using the following protocol: denaturation for 2 min at 95°C, followed by 30 cycles of 5 s at 95°C and 30 s at 65°C (for *Defensin 1*, *Vg* and *Ndufb2*) or 61.4°C (for *Cytb* and *COX1*). Oligonucleotide primers were used to detect the mRNA levels of *Def1* and *Vg*, which are crucial proteins for the insect immune system, and of *Ndufb2* (complex I), *Cytb* (complex III) and *COX1* (complex IV), which are mitochondrial proteins essential for the proper functioning of the ETS (Table S1). The relative quantification was calculated with the $2^{-\Delta\Delta C_t}$ method using *Actin-5C* and *40S ribosomal protein S18* as reference genes, and combining the warmest months (June, July and August) for the control group.

Hemolymph immune capacity

Collection and cell marking

Approximately 40–60 µl of hemolymph was collected as previously described (Borsuk et al., 2017) from 10 to 20 freshly collected bees (*N*=3 each month). To properly compare the hemolymph parameters of winter and summer phenotypes, the different samples were analysed together for months with average temperature <5°C (December, January, February and March for winter, *N*=12) and for months with average temperature >20°C (June, July and August for summer, *N*=9). All procedures were performed on ice. Insect culture medium 2X consisting of 40% Grace's insect medium (Sigma-Aldrich), 40% anticoagulant (98 mmol l^{-1} NaOH, 186 mmol l^{-1} NaCl, 1.7 mmol l^{-1} EDTA and 41 mmol l^{-1} citric acid buffer, pH 4.5) and 20% fetal bovine serum (Corning) was added to the hemolymph (1:1 v/v). The hemocytes' plasma membranes were labelled with 5 µg ml^{-1} wheat germ agglutinin Oregon Green (Molecular Probes, excitation=495 nm, emission=519 nm) according to the manufacturer's protocol. The concentration of hemocytes μl^{-1} was determined by flow cytometry (Attune NxT, Thermo Fisher Scientific).

In vitro phagocytosis capacity measurement

To assess the phagocytosis capacity, hemocytes were co-incubated in presence of fluorescent *Escherichia coli* (K-12 strain) BioParticles Alexa Fluor 594 conjugate (excitation=590 nm, emission=617 nm) as previously reported (Jougleux et al., 2021; Richardson et al., 2018). Briefly, 30,000 hemocytes labeled with 5 µg ml^{-1} Wheat Germ Agglutinin Oregon green were incubated with 30,000 *E. coli* beads (1:1). Cytochalasin D, a known phagocytic inhibitor, was also added as a control (100 mmol l^{-1}). Insect culture medium 1X was added to complete the volume to 100 µl and then incubated at room temperature for 1 h. The percentage of *E. Coli* associated with hemocytes was determined by flow cytometry.

Viability assay

Viability assays were performed by flow cytometry using a combination of Annexin V-Alexa Fluor™ 647 (Thermo Fisher Scientific) and Zombie-aqua™ (BioLegend) for labeling of hemocytes. Hemocytes were resuspended in 1X Annexin V buffer (Thermo Fisher Scientific). Cells were labeled with 1 µl of Zombie-aqua™ along with 1 µl of Annexin V-Alexa Fluor™ 647 for a total volume of 100 µl and incubated for 15 min in the dark and then processed by flow cytometry for cell viability assay. Hemocytes were identified as viable when cell labeling was negative for both Zombie-aqua™ and Annexin V staining. All data were normalized to non-treated (NT) labeled cells.

Statistical analysis

All statistical analyses were performed using R (version 3.6.0, R Foundation for Statistical Computing, Vienna, Austria). For respiration rates, mitochondrial ratios, enzymatic activities and transcript abundance, data were fitted to a linear model with month as fixed factor. Normality was verified with visualization of the residuals and homogeneity of variances was verified using the Levene's test, and data were transformed when required. A one-way ANOVA followed by a Tukey's *post hoc* test using the emmeans function (estimated marginal means) were then performed to estimate specific differences. In some cases, when ANOVA

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assumptions cannot be met after data transformation, a Kruskal–Wallis test followed by a Dunn's test was performed. All ANOVA *F*-values, d.f. and *P*-values obtained for all parameters measured and for each month are reported in Table S2, as well as chi-square values and *P*-values for the Kruskal–Wallis test, as appropriate. For hemolymph parameters, comparisons between winter (including December, January, February and March) and summer (including June, July and August) were performed using a Student's *t*-test after verifying normality and homogeneity of variances.

RESULTS

Mitochondrial respiration rates

For honeybees, shivering during winter and flying during summer are energetically costly, and are processes performed by the flight muscles found in the thorax. These muscle tissues are very dense in mitochondria and are at the core of substrate oxidation and ATP production used for muscle contraction.

Mitochondrial oxygen consumption of muscle tissue with the presence of pyruvate, malate and glutamate (CI-LEAK; Fig. 1A) showed a steady drop from September to March followed by an increase from March to June, and a stabilization during the summer months (from June to August). This resulted in a statistically significant decline in CI-LEAK observed in December, January and March compared with September, October, November, and from April to August, while February was significantly lower compared with September, June, July and August (Fig. 1A). When ADP was added to stimulate the oxidative phosphorylation process with complex I substrates (CI-OXPHOS, Fig. 1B), similar values were observed for September, October, November, April, May, June, July and August, which were all significantly higher than for

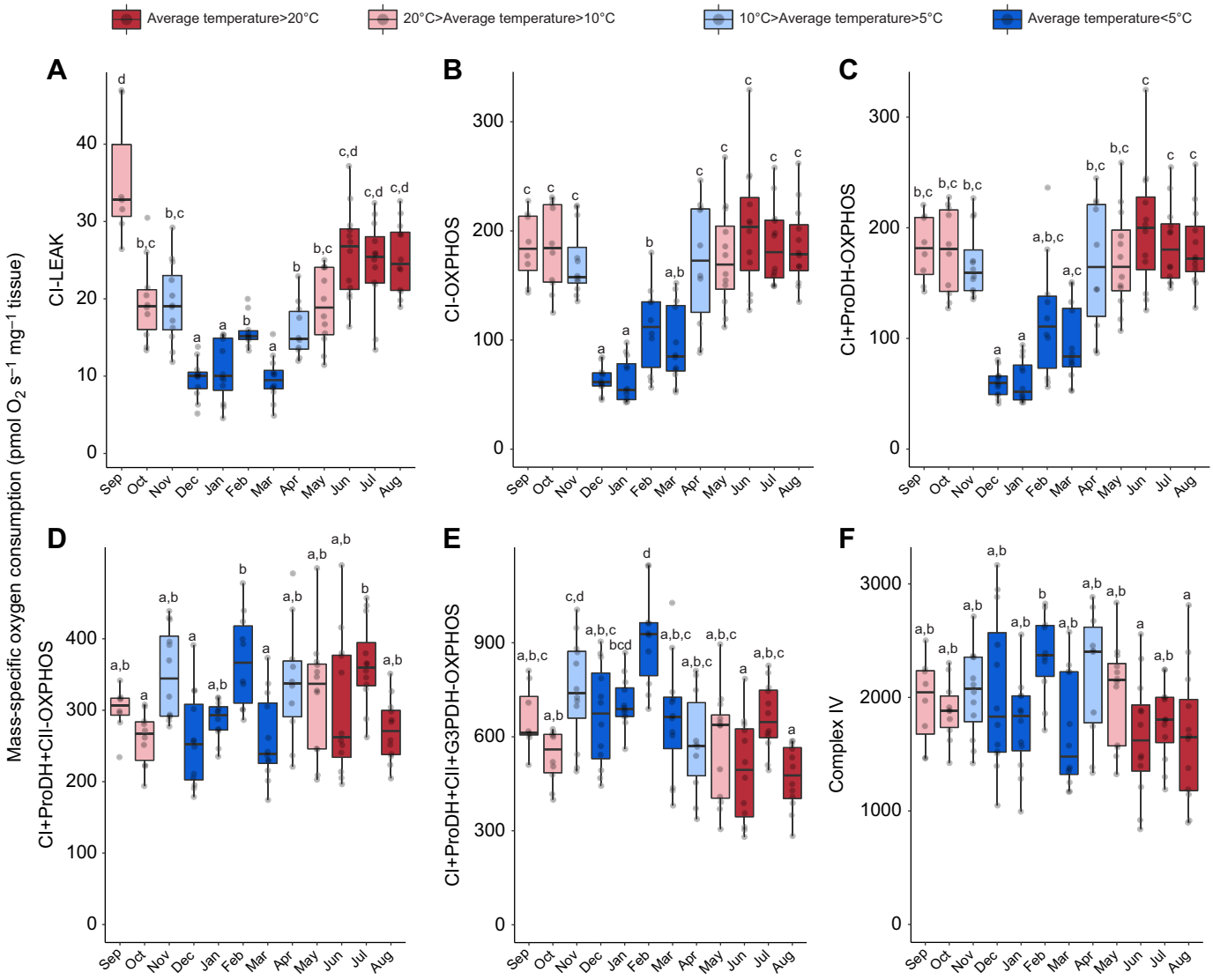

Fig. 1. Monthly mitochondrial respiration rates measured in thorax muscle of honeybees (*Apis mellifera*) from September 2020 to August 2021 (*N*=8–12). Mitochondrial oxygen consumption rates were measured in the presence of: (A) pyruvate, malate and glutamate (CI-LEAK); (B)+ADP (CI-OXPHOS); (C)+proline (CI+ProDH-OXPHOS); (D)+succinate (CI+ProDH+CII-OXPHOS); (E)+G3P (CI+ProDH+CII+mtG3PDH-OXPHOS); and (F) after inhibition of complexes I, II and III by rotenone, malonate and antimycin A, respectively, and addition of TMPD+ascorbate (CIV). CIV oxygen consumption rates were corrected in the presence of sodium azide to account for TMPD auto-oxidation rates. Box plot values consist of the median (center line) and interquartile range (IQR; upper and lower edges of box), and the whiskers correspond to maximum and minimum values <1.5× IQR (Tukey-style) for each month (*N*=8–12) with different letters denoting statistical differences between months (*P*<0.05) analysed with a one-way ANOVA followed by a Tukey *post hoc* test, or a Kruskal–Wallis test followed by a Dunn's test. Different colors indicate the average temperature range during each month.

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December, January, February and March (Fig. 1B). These results suggest that complex I-induced respiration was somehow repressed during winter. The exact same trend was also observed when proline was added as a substrate (CI+ProDH-OXPHOS; Fig. 1C), but with only December and January being statistically lower than September, October, November, April, May, June, July and August (Fig. 1C).

However, when succinate was added to stimulate complex II respiration (CI+ProDH+CII-OXPHOS; Fig. 1D), only slight variations were observed between each month, with only significantly lower rates detected between October, December and March compared with February and July (Fig. 1D). After addition of G3P, an important substrate for flying insects (Cormier et al., 2021), the same trend was observed in CI+ProDH+CII+mtG3PDH-OXPHOS respiration rates (Fig. 1E), with only small variations detected between the different months. Interestingly, the highest rate was reached during the coldest month, February (average temperature of −3.35±0.31°C; Fig. 1E and Fig. S1), which was statistically higher than all the other months except November and January, whereas the lowest rate was observed during the warmest month, August (average temperature of 23.91±0.26°C; Fig. 1E and Fig. S1), which was statistically different from November, January and February. Altogether, these results suggest a metabolic reprogramming occurring in winter bees, with generally higher capacities to oxidize succinate and G3P instead of complex I (NADH-linked) substrates such as pyruvate, malate and glutamate. For complex IV respiration rates (Fig. 1F), few significant differences were observed between months, with only February displaying higher values than June and August.

Mitochondrial ratios

The mitochondrial capacity to utilize different oxidative substrates influences mitochondrial oxygen consumption and ATP production and may indicate specific adjustments to temperatures. We thus calculated several ratios to estimate the contribution of each substrate to the mitochondrial respiration.

Although we detected a decreased oxygen consumption with complex I substrates (CI-OXPHOS; Fig. 1B), the coupling efficiency at the level of complex I (1–CI-LEAK/CI-OXPHOS), which is an indicator of the coupling between the transport of electrons from complex I and oxidative phosphorylation, was relatively constant throughout the year (Fig. 2A). In October, March and April, slightly but significantly higher CI coupling ratios were detected compared with September and January (Fig. 2A). Proline did not significantly contribute to mitochondrial respiration, as values calculated for proline contribution were close to 0 and did not vary significantly throughout the year (Fig. 2B).

In contrast, succinate contributed significantly to the mitochondrial respiration. Specifically, succinate doubled the oxygen consumption during the warmest months of the year in summer and autumn (succinate contribution ∼1.0 for September, October, May, June, July and August; Fig. 2C), and was tremendously augmented during the coldest months (December, January, February and March; Fig. 2C). This translated into significantly higher succinate contributions in December, January and February compared with all other months, while November and March also displayed significantly higher values than October, June and August (Fig. 2C). The same trend was observed for the G3P contribution, albeit this contribution doubled from September to November (G3P contribution ∼1.0; Fig. 2D), peaked during winter and then drastically decreased from April to August. Specifically, significantly higher G3P contribution ratios were detected in December, January, February and March compared with April, May, June, July and August (Fig. 2D). Moreover, the lowest values for this ratio were observed in April, June and August, which were significantly lower from all the other months except May and July (Fig. 2D). These results confirmed the metabolic reprogramming mentioned above, with higher contributions of succinate and G3P during winter. Moreover, it seems to indicate that G3P utilization might reflect the winter to summer phenotype transition.

Enzymatic activities

Owing to a strict honey diet during winter, nutrient availability is very limited in the honeybee cluster responsible for maintaining elevated temperature inside the hive. Consequently, winter bees must strictly rely on honey as fuel for mitochondrial oxidative phosphorylation, which is essential for shivering thermogenesis. Enzymatic activities fluctuated differently throughout the year (Fig. 3). In the glycolytic pathway, we evaluated hexokinase (HK), pyruvate kinase (PK) and lactate dehydrogenase (LDH; Fig. 3A–C). For HK, the warmest months, i.e. September, June, July and August, displayed the lowest activity (significant differences detected in September, June and August compared with December, January, April and May, as well as in July compared with October, December, January, February, April and May; Fig. 3A), whereas activity was the highest in December, January, April and May (significant differences detected in December, January and May compared with September, March, June July and August, as well as in April compared with September, November, March, June, July and August; Fig. 3A). For PK and LDH, the opposite trend was observed, with the lowest activities measured from October to April and the highest activities detected in September, June, July and August. Significant differences were observed from October to April compared with September, June, July and August for both enzymes (Fig. 3B,C). This suggests that metabolites from glycolysis are diverted before the formation of pyruvate, likely at the level of the dihydroxyacetone phosphate, which can be transformed to G3P for mitochondrial oxidation.

For pyruvate oxidation and the tricarboxylic acid cycle (TCA), we evaluated pyruvate dehydrogenase (PDH), citrate synthase (CS) and malate dehydrogenase (MDH), as well as aspartate aminotransferase (AAT), which is known to link amino acid metabolism to the TCA cycle. For PDH, we observed a trend similar to that for HK, with higher activities from December to May and lower activities from September to November and June to August (Fig. 3D), but significant differences were only detected in February, March, April and May compared with September, October, November, June, July and August (Fig. 3D). The same pattern was observed for AAT, CS and MDH (Fig. 3E–G), which resembled the patterns of PK and LDH: lower activities during the coldest months and higher activities during the warmest months (Fig. 3E–G). For AAT, September, June, July and August had higher values compared with October through April (Fig. 3E); and for CS and MDH, significantly lower values were observed for September, October, November, December February and March compared with January, April, May, June, July and August (Fig. 3F, G). Finally, we also evaluated the enzymatic activity of NADH dehydrogenase (Fig. 3H). Surprisingly, the enzyme activity did not parallel the results obtained for complex I-induced mitochondrial respiration, with activities displaying low variations between the different months. The lowest values were detected in June and July with significant differences in June compared with January through May, as well as in July compared with September, January, February, March, April and May (Fig. 3H).

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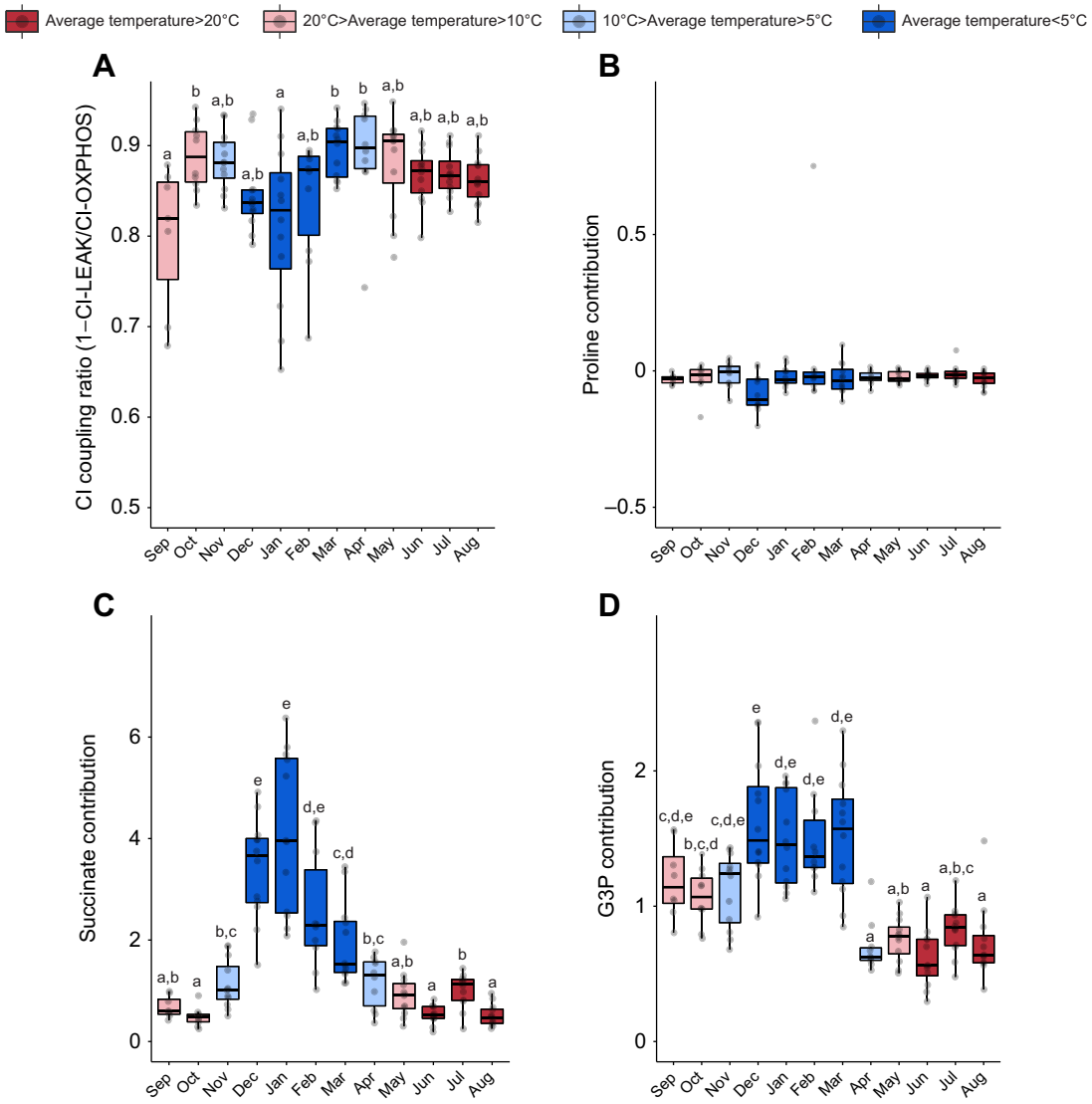

Fig. 2. Mitochondrial ratios calculated from mitochondrial respiration rates measured in thorax muscle of honeybees (*Apis mellifera*) from September 2020 to August 2021 (*N*=8–12). (A) CI coupling ratio=1−(CI-LEAK/CI-OXPHOS); (B) Proline contribution=(CI+ProDH-OXPHOS−CIc-OXPHOS)/CIc-OXPHOS; (C) Succinate contribution=(CI+ProDH+CII-OXPHOS−CI+ProDH-OXPHOS)/CI+ProDH-OXPHOS; and (D) G3P contribution=(CI+ProDH+CII+mtG3PDH-OXPHOS−CI+ProDH+CII-OXPHOS)/CI+ProDH+CII-OXPHOS). Box plot values consist of the median (center line) and IQR (upper and lower edges of box), and the whiskers correspond to maximum and minimum values <1.5× IQR (Tukey-style) for each month (*N*=8–12) with different letters denoting statistical differences between months ($P<0.05$) analysed with a one-way ANOVA followed by a Tukey *post hoc* test, or a Kruskal–Wallis test followed by a Dunn's test. Different colors indicate the average temperature range during each month.

Transcript abundance

Transcript abundance of genes encoding subunits of complexes I, III and IV (*Ndufb2*, *Cytb* and *COX1*, respectively) of the ETS were also evaluated. The relative expressions of all three genes were only slightly modulated throughout the year (reaching up to ∼1.5- to 2-fold difference; Fig. 4A–C). For *Ndufb2*, the highest transcript abundances were observed in February and March, which were statistically higher than November, April, May, July and August (Fig. 4A). However, for *Cytb* and *COX1*, little variation in transcript abundance was detected throughout the year with some significant differences detected between months, but no clear pattern in relation to the temperatures of the months (Fig. 4B,C).

Vg and *Def1* are genes encoding for important proteins central to the honeybee's immune system. Both genes showed higher mRNA levels during colder months compared with warmer months (Fig. 4D,E). *Vg* transcript abundance displayed a steady increase from September to February–March (up to ∼8.5-fold increase), followed by a sharp decline in April, and few variations were then detected between May and August (Fig. 4D). For *Def1*, the same trend was observed, with a steady increase in winter (albeit with a slight decrease in February) that was, however, maintained in April (up to ∼7.5-fold increase), followed by a decline in May that persisted until August (Fig. 4E).

Phagocytosis and hemocyte viability assay

Hemocytes are the key immune cells found in the honeybee's hemolymph and the first line of defense against pathogens. Both their viability and their phagocytic capacity represent an estimate of their capacity to respond to pathogen infection and to mount an appropriate cellular response to this infection. For the evaluation of these parameters, the samples collected during the coldest months (December to March) and the warmest months (June to August)

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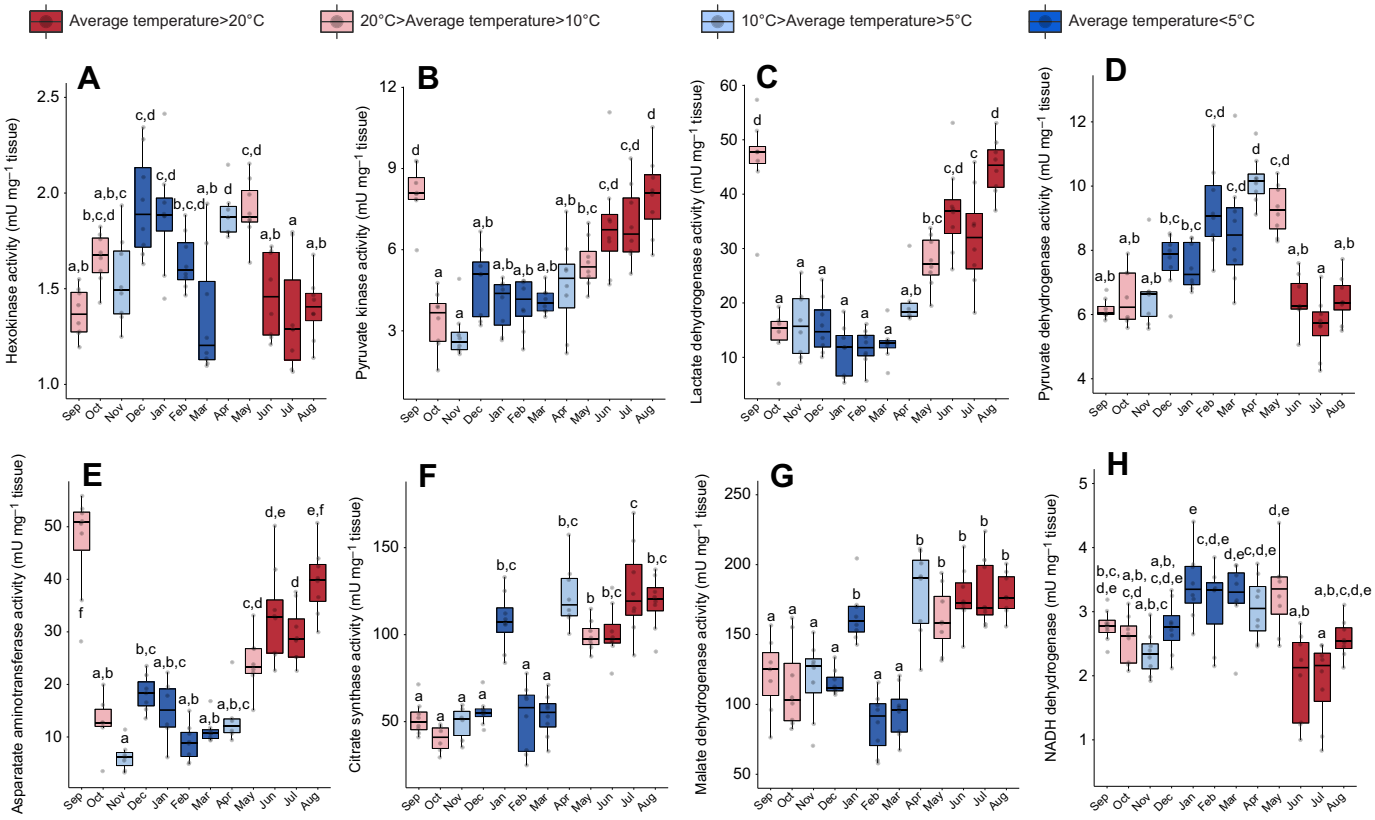

Fig. 3. Activities of enzymes involved in different metabolic pathways measured in thorax muscle of honeybees (*Apis mellifera*) from September 2020 to August 2021 (*N*=6–8). (A) Hexokinase; (B) Pyruvate kinase; (C) Lactate dehydrogenase; (D) Pyruvate dehydrogenase; (E) Aspartate aminotransferase; (F) Citrate synthase; (G) Malate dehydrogenase; and (H) NADH dehydrogenase. Enzyme activities were measured at 35°C. Box plot values consist of the median (center line) and IQR (upper and lower edges of box), and the whiskers correspond to maximum and minimum values <1.5× IQR (Tukey-style) for each month (*N*=6–8) with different letters denoting statistical differences between months (*P*<0.05) analysed with a one-way ANOVA followed by a Tukey *post hoc* test, or a Kruskal–Wallis test followed by a Dunn's test. Different colors indicate the average temperature range during each month.

were analysed together. Phagocytosis capacity, estimated by the capacity of hemocytes to engulf fluorescent beads coated with *E. coli*, was significantly higher during winter (~7-fold increase) than during summer (*t*-test, $P<0.001$; Fig. 5A). The viability of hemocytes (measured via percentage of apoptotic cells) was also slightly but significantly higher during winter (~1.5-fold increase) compared with summer (*t*-test, $P=0.028$; Fig. 5B).

DISCUSSION

In this study, we evaluated mitochondrial respiration, enzyme activity, transcript abundance of genes involved in the ETS and in immunity, as well as the viability of hemocytes and their phagocytosis capacity in honeybees every month for a year to compare the physiological and cellular changes occurring between summer and winter. We hypothesized that a major metabolic reprogramming at the mitochondrial level occurs in winter individuals, favoring shivering thermogenesis. We also hypothesized that owing to this metabolic switch directing nutrients towards muscle contractions, the capacity for mounting an immune response would be decreased in winter.

Surprisingly, complex I-induced respiration was repressed in honeybees during winter (Fig. 1A,B), suggesting that NADH-linked substrates derived from glycolysis and the TCA cycle are not actively participating in mitochondrial respiration. This could be due to a deficiency at the complex I level. However, neither *Ndufb2* transcript abundance (Fig. 4A) nor NADH dehydrogenase enzymatic activity (Fig. 3H) displayed this decrease in winter, and the coupling ratio at the level of complex I remained constant throughout the year (Fig. 2A). Thus, the most parsimonious explanation for these results is that complex I-induced respiration with NADH-linked substrates is regulated through post-translational modifications, pointing to a regulatory switch. The results obtained for NADH dehydrogenase activity (Fig. 3H) might be due to the loss of such modification during the processing of samples for enzymatic activity of tissues compared with the gentler permeabilization process used for measuring mitochondrial respiration. In liver and brown adipose tissue of hibernating thirteen-lined ground squirrels, it has been shown that although complex I-induced mitochondrial respiration in isolated mitochondria was drastically reduced in torpor owing to post-translational modifications, enzymatic activities of complex I in homogenized tissues were unchanged compared with interbout euthermia ground squirrels (Mathers and Staples, 2019; Mathers et al., 2017; McFarlane et al., 2017). Moreover, it has been shown that complex I can transition from an active to a dormant conformation in several organisms when temperature varies or in tissues lacking oxygen (Babot et al., 2014; Bundgaard et al., 2018, 2020; Chouchani et al., 2014), albeit not specifically in insect species. It is thus tempting to suggest that the decreased complex I-induced respiration is caused by the conditions experienced by the cluster during winter (temperature variation and/or oxygen availability). Another possible explanation (not mutually exclusive from the

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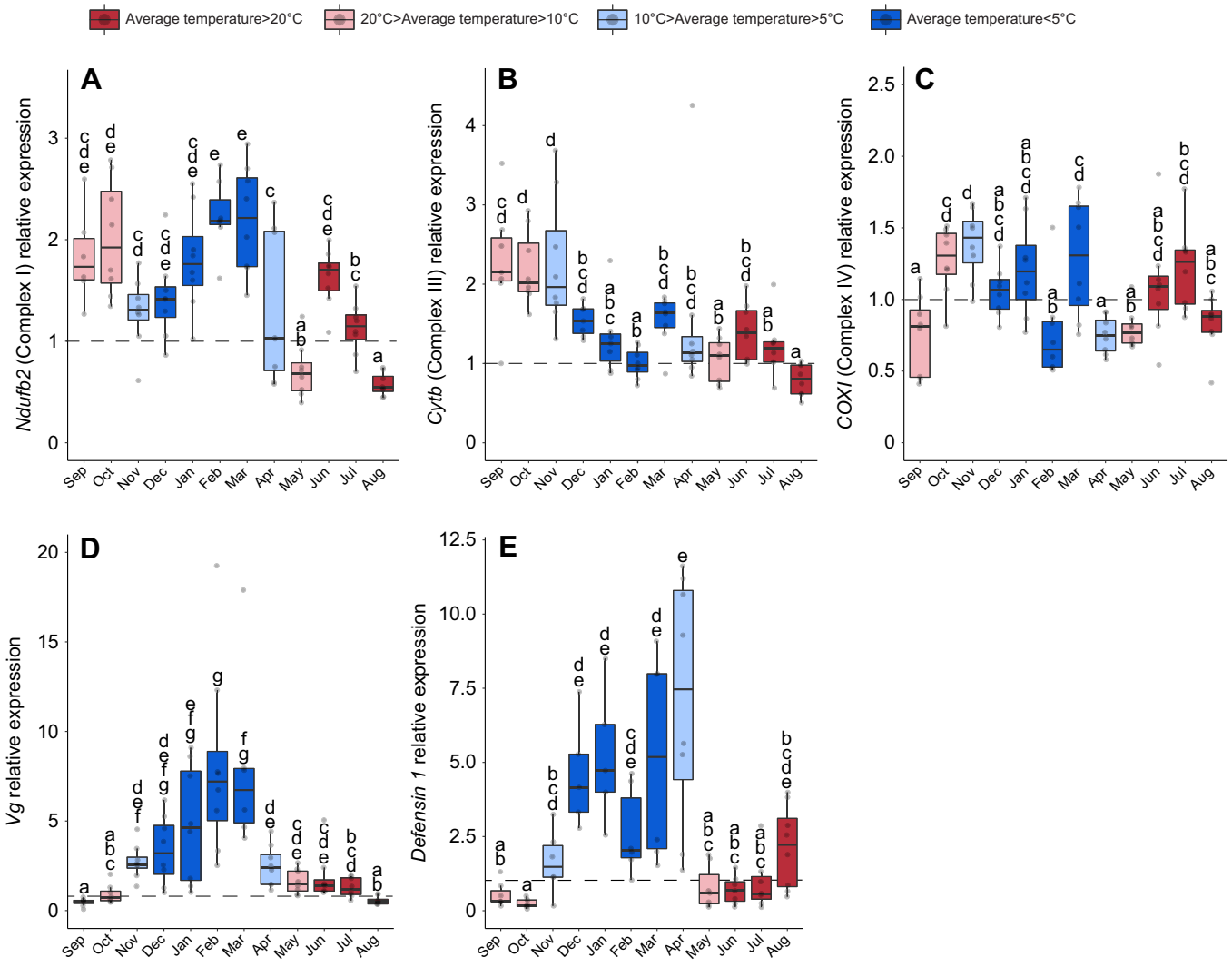

Fig. 4. Monthly transcript abundance of immune and mitochondrial genes measured in RNA extracted from thorax muscle of honeybees (*Apis mellifera*) from September 2020 to August 2021 (*N*=5–8). Relative transcript abundance of (A) *Ndufb2* (complex I subunit), (B) *Cytb* (complex III subunit), (C) *COXI* (complex IV subunit), (D) *Vitellogenin* and (E) *Defensin-1*. Each bar represents the mean value±s.e.m. of the relative quantification calculated with the $2^{-\Delta\Delta C_t}$ method using *Actin-5C* and *40S ribosomal protein S18* as reference genes, and combining the warmest months (June, July and August) for the control group. Box plot values consist of the median (center line) and IQR (upper and lower edges of box), and the whiskers correspond to maximum and minimum values <1.5× IQR (Tukey-style) for each month (*N*=5–8) with different letters denoting statistical differences between months (*P*<0.05) analysed with a one-way ANOVA followed by a Tukey *post hoc* test, or a Kruskal–Wallis test followed by a Dunn's test. Different colors indicate the average temperature range during each month.

previous one) is that the mitochondrial NADH-producing enzymes are limiting the supply of NADH. Indeed, we observed that CS, MDH and AAT (Fig. 3E–G), which all participate to increase the flux of metabolites in the TCA cycle and thus the production of mitochondrial NADH, are all decreased during winter. However, this was not the case for PDH (Fig. 3F), which controls the flux of acetyl-CoA, as the activity of this enzyme is increased during winter.

Honeybees are thought to be strict users of carbohydrates for metabolic fuel; however, it has been suggested that amino acids such as proline can be oxidized and used to fuel insect metabolism, specifically in hymenopterans (Teulier et al., 2016). Here, proline is not a good substrate for honeybees whatever the season (Figs 1C and 2B). On the contrary, succinate contributed the most compared with all the other substrates tested for mitochondrial respiration, especially in winter individuals, with a 200–500% increase in oxygen consumption during the coldest months (Fig. 2C). Succinate is known to accumulate during hypoxia in several organisms including insects (albeit not specifically in winter honeybees) (Colinet and Renault, 2012; Harrison et al., 2018; Hoback and Stanley, 2001; Marden et al., 2021). The increased succinate contribution observed in winter honeybees highlights the important role of this substrate for the winter cluster and might indicate an influence of oxygen availability.

G3P is also known to support mitochondrial respiration in insects, especially when a complex I dysfunction occurs (Cormier et al., 2019, 2021; Jørgensen et al., 2021; Menail et al., 2022; Pichaud et al., 2019). We also observed an increased capacity for G3P oxidation in winter honeybees when complex I-induced respiration was at its lowest (Figs 1E and 2D). Thus, similarly to succinate, G3P seems to be an important oxidative substrate for winter honeybees. Interestingly, we also showed that HK, the first enzyme of glycolysis, is increased during winter, contrary to PK and LDH, the last enzymes of the pathway (Fig. 3A–C). These results,

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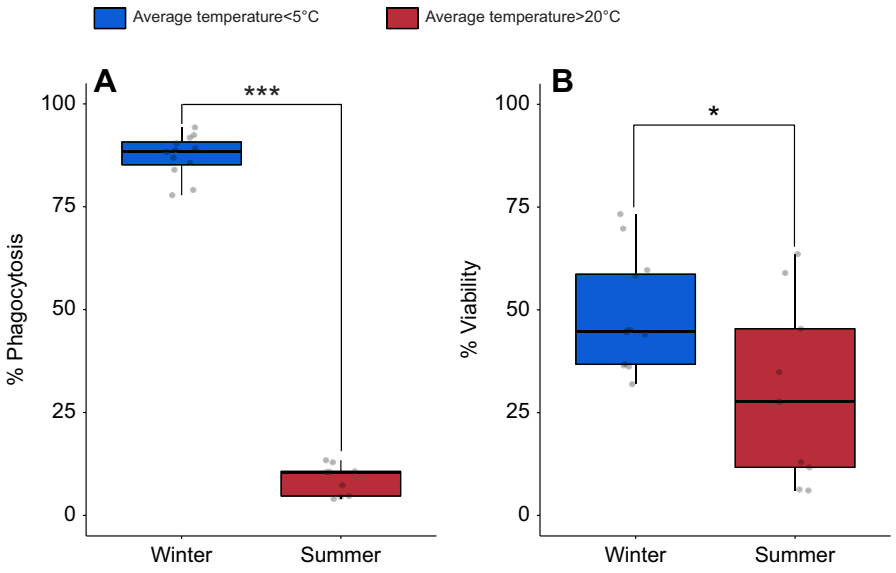

Fig. 5. Phagocytosis capacity and cell viability measured in hemocytes of honeybees (*Apis mellifera*) during the winter months (December to March, *N*=12) and the summer months (June to August, *N*=9). (A) Percentage of phagocytosis estimated by the capacity of hemocytes to engulf fluorescent beads coated with *E. coli*. (B) Hemocyte viability measured by flow cytometry using a combination of Annexin V-Alexa Fluor™ 647 (ThermoFisher Scientific) and Zombie-aqua™ (BioLegend) for labeling. Box plot values consist of the median (center line) and IQR (upper and lower edges of box), and the whiskers correspond to maximum and minimum values <1.5× IQR (Tukey-style) for either winter or summer (*N*=12 and *N*=9, respectively). Differences between winter and summer were obtained following a Student's *t*-test (*0.01<*P*<0.05; ***P*<0.001).

combined with the decreased complex I-induced respiration, suggest that the utilization of carbohydrates via glycolysis to sustain mitochondrial metabolism would have to slow down, resulting in the accumulation of metabolites upstream of PK. However, this should be resolved by the observed increased oxidation of G3P, which is an oxidative substrate that can be formed by glycolysis via the conversion of dihydroxyacetone phosphate to G3P. Thus, this will allow the constant use of the carbohydrates contained in honey as metabolic fuel in winter honeybees. It is also possible that the differences in respiration rates observed between summer and winter bees reflect differences in mitochondrial content between individuals. Indeed, CS activity, which is often used as a marker of mitochondrial content, is decreased during winter (Fig. 3F). However, another marker of mitochondrial content, complex IV maximum capacity, is only slightly changed during winter. It has been shown in honeybees that complex IV operates close to its maximum catalytic capacity, and that the measurement of its activity reflects a good estimate of cristae surface area (Suarez, 2000; Suarez et al., 2000). Although we cannot confirm whether the differences in respiration rates are indeed due to differences in mitochondrial content, our results on complex IV maximum capacity and *COXI* transcript abundance suggest that mitochondrial content is similar between summer and winter honeybees.

The increased capacity to oxidize succinate and G3P for mitochondrial respiration instead of NADH-linked substrates by complex I has important consequences in terms of ATP production. As neither complex II nor mtG3PDH directly participate in the pumping of protons from the matrix to the intermembrane space, this should theoretically diminish the net amount of ATP produced by OXPHOS. In honeybees raised in the laboratory, it has been shown that succinate oxidation is not associated with membrane potential generation and oxidative phosphorylation (Syromyatnikov et al., 2019). Similarly, G3P-supported OXPHOS was shown to be inefficient from 15 to 35°C, as P:O ratios were low at these temperatures in bumblebees (Masson et al., 2017). Thus, combined with the decreased complex I-induced respiration, this suggests that shivering thermogenesis, which is a process requiring muscle contraction and a continuous supply of ATP, might not be the principal mechanism allowing the winter cluster to maintain the temperature inside the hive. Other cellular processes such as increased activity of sarcoplasmic reticulum Ca^{2+}-ATPase (SERCA) pumps (Nowack et al., 2017) or futile cycles (Masson et al., 2017; Staples et al., 2004) might be involved, albeit both also rely on ATP supply. In contrast, non-shivering thermogenesis might be a good candidate to explain the high temperature maintained by the winter cluster through increased utilization of succinate and G3P and activation of uncoupling proteins. However, such processes still need to be characterized in insect species, and may be a focus of further research. Nevertheless, the switch in mitochondrial oxidative substrate utilization occurring during winter appears to be a specific mechanism for honeybees required for thermoregulation.

In line with previous studies (Amdam et al., 2004; Salmela et al., 2016), we detected a significantly higher level of *Vg* transcript abundance in winter bees compared with summer bees (Fig. 4D). Vg is an important protein for immunity, and it has been hypothesized that the high Vg level of winter bees is involved in their increased life span through effects on cellular immunity, storage protein concentration and antioxidant function (Amdam and Omholt, 2002; Amdam et al., 2004). Our results showing a steady increase in *Vg* transcript abundance in winter that peaked at the beginning of spring indicate that this period is crucial for preparing the immune system of honeybees for higher external temperatures and potentially higher pathogen exposure (Gisder et al., 2017), and support that Vg is a key element for colony fitness (Lourenço et al., 2019; Ricigliano et al., 2018; Smart et al., 2016). Similarly, the observed significant and steady increased mRNA levels of *Def1* in winter bees (Fig. 4E) are consistent with prior studies (Barroso-Arévalo et al., 2019; Dostálková et al., 2021) and corroborate the results on *Vg*. Hence, contrary to our hypothesis, these results indicate that important modulators of the immune response are increased in winter bees.

This is also confirmed by our results on the physiology of hemocytes. In honeybee hemolymph, these specialized cells are responsible for the cellular immune response, which consists of nodulation, encapsulation and phagocytosis. Our results show that phagocytic capacity of hemocytes, which has also been suggested to be primed by Vg (Gätschenberger et al., 2013; Hystad et al., 2017; Salmela et al., 2015), is increased during winter (Fig. 5A). However, it has previously been shown that nodulation and encapsulation are increased in summer bees (Gätschenberger et al., 2013; Steinmann et al., 2015). One possible explanation for this discrepancy could be

that phagocytosis is the first immune process used for bacterial elimination during winter and therefore compensates for the lack of both nodulation and encapsulation during this season, which also aligns with our results on *Vg* transcript abundance. Gätschenberger et al. (2013) also studied the immune responses of summer and winter bee populations maintained under laboratory conditions and found that winter bees reduced the number of viable injected *E. coli* faster than summer bees. Moreover, a recent study (Dostálková et al., 2021) found the same tendency, as antimicrobial activity was much more effective during winter compared with summer in honeybees. In line with our results, this confirms that the capacity to mount a cellular immune response is increased during winter. We also characterized cell viability of hemocytes, which was significantly higher in winter bees compared with summer bees. In previous studies that measured the total number of hemocytes in honeybees across the entire year, no significant differences between summer and winter populations were detected (Dostálková et al., 2021; Kunc et al., 2019). Thus, although cell abundance does not fluctuate throughout the year, cell viability is increased during winter. We suggest that, owing to overexpression of AMPs such as *Def1* and *Vg* during winter in honeybees, antimicrobial activity and phagocytosis are enhanced, which in turn allows immune cells to survive through winter. The high mortality rate of colonies during winter thus might not be due to a failure of the cellular response to pathogens (at least when a colony is not previously infected).

Our initial hypothesis that the metabolic needs for thermogenesis are impacting the immune system owing to metabolic fuels being redirected to sustain muscle contractions is erroneous, as immunity seems to be privileged during winter. An explanation for this would be that thermogenesis during winter is not as energetically costly as we first predicted compared with foraging during summer. Although it has been shown that the metabolic rate of clustering honeybees can increase by up to 25 times during winter and increases steadily with air temperatures below 5°C (Southwick and Heldmaier, 1987), owing to a lack of proper comparisons of metabolic rates in summer and winter honeybees, we cannot confirm which process (foraging or shivering) is the more costly. It has also been shown in insects that mounting an immune response can raise the metabolic rate by up to 28% (Ardia et al., 2012; Freitak et al., 2003), which can be considered moderate compared with the increased metabolic rate associated with thermogenesis. However, another important aspect to consider is that a systemic metabolic switch may occur in the fat body of winter honeybees that favors the synthesis of AMPs and vitellogenin instead of directing the energy/nutrient flow to the skeletal muscle for shivering. Thus, an increased investment in immunity likely entails specific energetic and corresponding physiological costs. Our results suggest that despite lower probability of external infections of the bee cluster during winter (Gätschenberger et al., 2013), honeybees may invest more energy in immunity (with higher phagocytic activity linked to *Vg* transcript abundance) to ensure the integrity of the cluster (which perform thermogenesis through winter), and thus the survival of the colony. In contrast, summer bees will invest more in foraging than in immunity, as these bees are likely more expendable for the colony. Such a trade-off between immune function and metabolism in honeybees warrants further investigation.

In summary, our study shows that an important metabolic reprogramming occurs in honeybees during winter, with a switch in the utilization of mitochondrial substrates. Specifically, a reduced reliance on NADH-linked substrates through complex I (which might be due to post-translational modifications) and an important increased contribution of succinate and G3P to mitochondrial respiration indicate a specific phenotypic change during winter, with both succinate and G3P being important substrates for the colony thermoregulation. This substrate switch indicates that different thermoregulating strategies might occur in overwintering honeybees. We also showed that, contrary to our predictions, the immune system is enhanced during winter and thus that a failure of the cellular response to the pathogen is not a likely candidate explaining the high mortality rates in winter. To the best of our knowledge, this is the first study investigating metabolism while simultaneously characterizing immune response in honeybees during a 12-month period. Our results thus have an important impact on the comprehension of honeybee metabolic and immune system physiology during seasonal transition. As extreme weather patterns become more frequent owing to climate change, this study will help us to better understand the challenges that essential and economically important insects such as honeybees will face in the future. Investigating as-yet uncharachterized thermoregulating strategies as well as determining post-translational modifications and their functional effects in mitochondria are interesting research avenues worth exploring to understand the endothermic behavior of honeybees during winter.

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Competing interests
The authors declare no competing or financial interests.

Author contributions
Conceptualization: S.B.C., L.H.B., N.P.; Methodology: S.B.C., A.L., L.H.B., N.P.; Validation: S.B.C., N.P.; Formal analysis: S.B.C., A.L., L.H.B., N.P.; Investigation: S.B.C., A.L., L.H.B., N.P.; Resources: L.H.B., N.P.; Writing - original draft: S.B.C., N.P.; Writing - review & editing: S.B.C., A.L., L.H.B., N.P.; Supervision: N.P.; Funding acquisition: L.H.B., N.P.

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Fig. S1. External temperatures recorded by the smart hives from the 1st of September 2020 to the 1st of September 2021. Average temperatures are given for each month above the complete temperature profile.

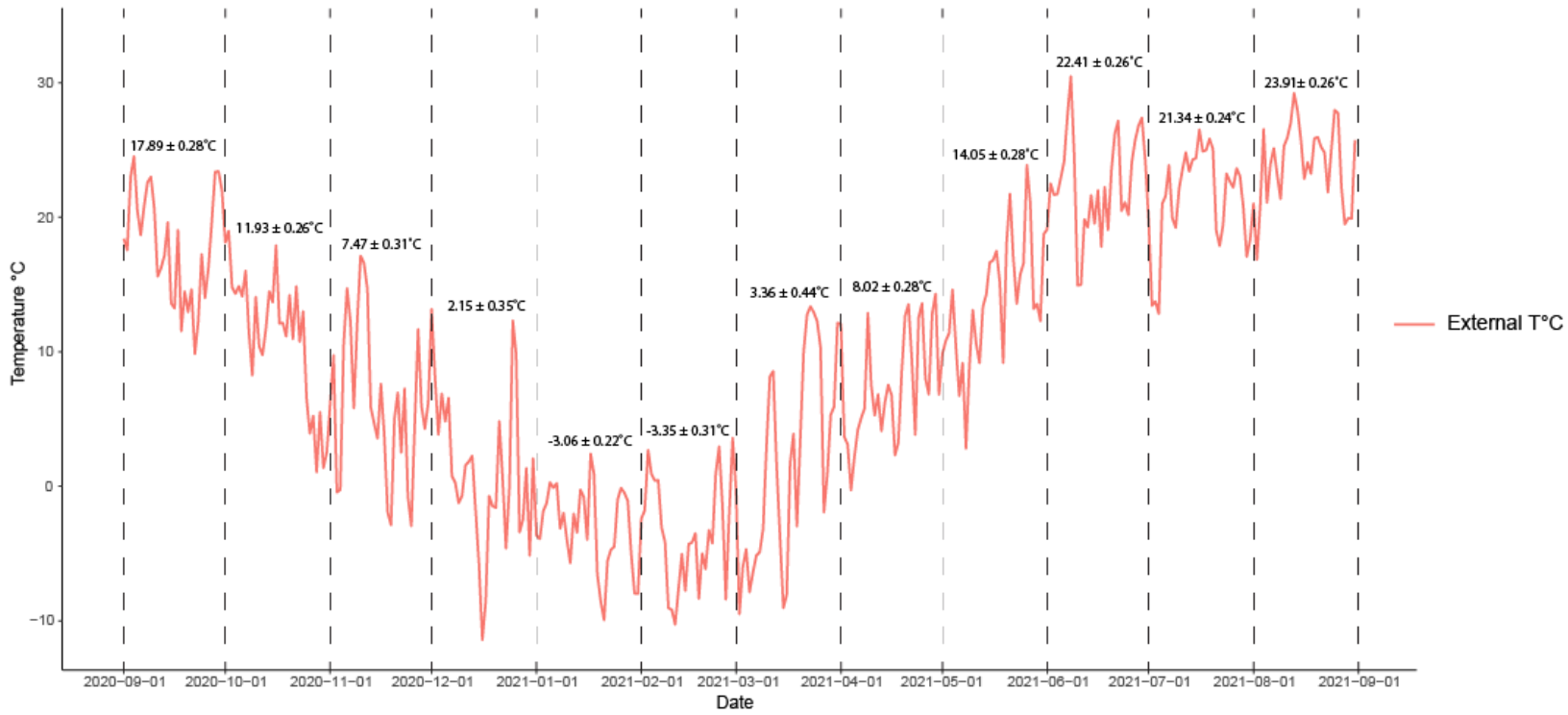

Table S1. Primer sequences used for q RT-PCR.

Gene name	Foward (5’ to 3’)	Reverse (5’ to 3’)	Product lenght (bp)	Accession number
***Defensin 1* (*Def 1*)**	TGCGCTGCTAACTGTCTCAG	AATGGCACTTAACCGAAACG	119	XM_017050425.2
***Vitellogenin* (*Vg*)**	TTTCTCGAACGGAAGGACGG	CCTGATCTCGAACGGTTGCT	118	XM_001122505.5
***NADH dehydrogenase 1 beta subcomplex 2* (*Ndufb2*)**	TTGGGTGGAATAATGTGGTGGT	TCATCTGTCCAATCTGCTGG	104	NM_001163463.1
***Mitochondrial cytochrome b* (*Cytb*)**	TATGTACTACCATGAGGACAAATATC	ATTACACCTCCTAATTTATTAGGAAT	485	GU979493.1
***Mitochondrial cytochrome c oxidase subunit 1* (*COX1*)**	ACCTGATATAGCATTCCCCCG	TCATCCAGTTCCTGGTCTTGG	112	LC626902.1
***Actin-5C* (*Act*)**	TGCCAACACTGTCCTTTCTG	AGAATTGACCCACCAATCCA	156	XM_017059067.2
***40S ribosomal protein S18* (*S18*)**	GATTCCCGATTGGTTTTTGA	CCCAATAATGACGCAAACCT	149	XM_625101.6

Table S2. F and P-values obtained after one-way ANOVA on the different parameters measured in Honeybees (Apis mellifera). When the ANOVA assumptions cannot be met after data transformation, a Kruskal-Wallis test was performed and X^2 and P-values are reported.

Parameters measured	Residual *Df*	Month *Df* = 11	
		F or X^2 value	P value
Mitochondrial respiration rates			
CI-LEAK	121	F = 26.74	< 0.001
CI-OXPHOS	122	F = 25.15	< 0.001
CI+ProDH-OXPHOS	NA	X^2 = 78.07	< 0.001
CI+ProDH+CII-OXPHOS	NA	X^2 = 39.70	< 0.001
CI+ProDH+CII+G3PDH-OXPHOS	122	F = 7.60	< 0.001
Complex IV	122	F = 2.42	0.009
Mitochondrial ratios			
CI coupling ratio	121	F = 3.58	< 0.001
Proline contribution	NA	X^2 = 13.36	0.27
Succinate contribution	122	F = 33.48	< 0.001
G3P contribution	122	F = 19.81	< 0.001
Enzymatic activities			
HK	84	F = 9.41	< 0.001
PK	84	F = 16.44	< 0.001
LDH	80	F = 37.43	< 0.001
PDH	84	F = 17.79	< 0.001
AAT	75	F = 36.52	< 0.001
CS	80	F = 36.02	< 0.001
MDH	83	F = 19.062	< 0.001
NADH dehydrogenase	83	F = 7.80	< 0.001
Gene expression			
Vg	82	F = 19.32	< 0.001
Def1	68	F = 12.75	< 0.001
Ndufb2	81	F = 15.73	< 0.001
Cytb	81	F = 9.22	< 0.001
COX1	84	F = 6.11	< 0.001