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NMR Biomarkers of Cold Stress Resistance in Honey Bees

Diploma Thesis

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Declaration

I declare that the Diploma Thesis titled “NMR Biomarkers of Cold Stress Resistance in Honey Bees” is my own work and all the sources I cited in it are listed in Bibliography.

Prague, 11th April, 2019 Signature _____

Acknowledgement

I would like to offer my gratitude to the faculty and school staff of the Czech Universtiy of Life Sciences Prague who have helped me complete this thesis. I have special thanks for my thesis advisor Doc. Ing. Jaroslav Havlík, Ph.D., for his supprot and guidance for this thesis, as well as Pavel Mrňa, Dr. Alberto Jan Ruiz Del Valle, Dr. Martin Kamler. I would also like to thank my husband, family, and all of my friends who have encouraged me along the way.

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Summary

Honey bees fall into a comatose state in the ambient temperature ranges of 9 to 12°C with cold death occuring at -2 to -6°C. This study placed honey bees (*Apis mellifera)* into low temperature (7.6°C), and observed honey bees falling into a comatose state. We found that approximately the half of the honey bees fell into a chill coma, and the others did not fall into a comatose state but remaining alert. To determine the metabolic differences between honey bee group which fell into a comatose state and the honey bee group which did not fall into a comatose state, the present study used ^{1}H NMR spectroscopy to measure the metabolomic responses.

This study detected 11 of significant metabolic features: AMP, acetate, carnitine, glutamine, methylguanidine, NAD+, o-phosphocholine, phenylalanine, putrescine, trehalose, and β-alanine. Trehalose, as well as amino acids were higher in both honey bees groups. However, all of the significant compounds in the non-coma honey bee group (except for phenylalaine) was 1.2 times to 2.4 times higher than in the honey bee group which fell into a comatose state. The highest compound was dissacharide, trehalose which is known as a blood sugar compound in insects, and is also an important cryoprotectant for insects. Amino acids, alanin and proline which are typical energy precursors of insects, were not detected in the present study. However, glutamine concentrations were significant in the non-coma honey bees group as well as carnitine which is crucial for fatty acid oxdiation.

Key words: NMR analysis, chill-coma, cold resistence, honey bee, apis mellifera, cold-hardening, overwintering, cold tolerance, trehalose, amino acids

Content

1 Introduction

Historically, humanity has been using honeybee products for at least 9000 years, and has been producing them in agriculture for at least 4000 years (Roffet-Salque et al., 2015). Presently, honey bee keeping has become a crucial part of modern agriculture, not only producing honey, wax, and other traditional products, but is now helping farmers proactively pollinate crops to increase yield. For example, during the almond pollination season in California, more than 70% of all commercial honey bee colonies in the U.S are used in almond pollination, this demand continues to rise as more farmers realize the potential of this practice (H. Lee et al., 2018). Honey bees play an important part in not only the almond industry, but also in the stone fruits, vegetables across agriculture industry (Morse et Calderone, 2000).

Despite all the importance, honey bee population in the USA (59% loss of colony from 1947 to 2005) and Europe (25% loss in central Europe from 1985 to 2005) declines. Several factors are affected on honey bee colony loss; habitat loss, agrochemicals, pathogens, climate changes (Potts et al., 2010). Especially annual winter colony losses are keep showing in worldwide (22 to 36% of overwintering loss in the USA, 10 to 35% of overwintering loss in Europe) (VanEngelsdorp et al., 2008; vanEngelsdorp et Meixner, 2010).

Honey bee colony have an excellent adapting skill under the severe cold. Clustering, shivering the thorax flight muscle to generate heat. Thus, they can keep the core of the hive temperature up to 36°C (Bujok et al., 2002). A study reported that a honey bee colony have survived overnight under at -80°C (Southwick, 1987). However, individual honey bees cannot tolerate in harsh cold as the colony does. In certain low temperature, they fall into a comatose state, it is called chill coma. (Free et Spencer-Booth, 1960) observed that honey bees fall into a chill coma at 9 to 12°C, and occur cold death at -2 to -6°C.

Previous, yet unpublished study conducted by our team found that winter bees, which are born in the hive in the mid september, with the aim to guarantee a colony survival throughout the winter and have extended lifespan of nearly 200 days in comparison to normal bees living 42 days (reference) have significantly higher concentration of compounds such as trehalose, fructose, proline, glycine, and others than those found in summer bees. Also, metabolic fluctuations have seen over the course of the year. Based on the research, this study assumed that there would be metabolic differences in honey bees who fall into a chill coma and those which do not fall into a chill coma. Within this research, 120 honey bees were collected, exposed to cold temperature. The honey bees divided chill coma and non-chill coma were

analyzed by the ^{1}H NMR, and 11 statistically significant compounds were determined for the result.

2 Hypothesis and Objectives

The objective of this study was to compare the metabolic differences in 68 honey bees which showed active moving (non-chill coma) and other 52 those which did not moving (chill coma state) under the cold exposure (at 7.6°C) by NMR analysis.

It was hypothesized that there would be different metabolic features in between the honey bee group which fell into a chill coma and the honey bee group which did not fall into a chill coma.

3 Literature review

3.1 Honey bee overwitering

The honey bee is one of few insect species that has adapted to winter condition without entering a dormant stage. Their specialized behavior and physiological features for surviving winter help keep the colony maintained (Winston, 1987). However, since the massive colony losses in the U.S. in the winter of 2006-2007, bee keepers in the U.S. have been reporting annual winter colony losses. In recent decades, overwintering losses in the U.S. have consistantly been reported at about 22 to 36% (Kulhanek et al., 2017; K. V. Lee et al., 2015; Seitz et al., 2015; Steinhauer et al., 2014; VanEngelsdorp et al., 2008). Out of the top ten reasons for colony death, bee keepers responded “Poor winter” as one of the top factors for overwintering losses (Seitz et al., 2015; VanEngelsdorp et al., 2008). Overwintering colony losses in Europe show similar results. A study showed that 10 to 35% of colony winter losses were reported in 2007/2008 in Europe. Denmark, France, Slovenia, and United Kingdom ranked the highest (about 30% losses) among the 15 European countries studied (vanEngelsdorp et Meixner, 2010).

3.1.1 Factors affecting overwintering colony losses

There are many factors which impact colony losses during the winter. According to U.S. annual surveys, causes of overwintering mortality differs by operation type. Backyard bee keepers have generally indicated starvation, and weak colonies in the fall. Whereas commercial bee keepers indicated queen failure, pesticides, and varroa mites (Seitz et al., 2015; Steinhauer et al., 2014). A study from (Döke et al., 2015) showed that varroa mites, viruses (namely Deformed Wing Viruses, Israeli Acute Paralysis Virus, and Acute Bee Paralysis Virus), the genetic background of the colony, colony size and nutritional stores, and pesticides correlated with colony survival during the winter.

3.1.2 Overwintering behavior of honey bees

The most important challenge for the honey bee colony is getting through the cold winter. When the ambient temperature drops below 15-18°C, bees start to cluster to save heat in the colony (Moritz et Southwick, 1992; Winston, 1987). However, when the ambient temperature decreases to -5°C or lower, bees generate through a process different than clustering to maintain the temperature in the hive. Honey bees generate heat through shivering flight muscles in the thorax without moving their wings and increasing their metabolic rate to

similar level as flying bee (Winston, 1987). Thanks to this work, the brood rearing area which is located in the core of the nest, can be kept temperature at up to 36°C (Bujok et al., 2002), in ambient temperature ranges of -40 to 40°C (Winston, 1987). The secret to maintain the warm temperature is transferring high thoracic temperature to the cells. Bees firmly press their thoraxes onto the surface of the brood caps. This behavior warms the brood caps up to 3°C within 30 minutes, and this heat flow transfers to surrounding cells (Bujok et al., 2002; Kleinhenz, 2003). Honey bees convert glucose from glycogen stored in flight muscles, and glucose will be used for heat generating metabolism for 10-20 minutes. After the glycogen stores runs out, trehalose from haemolymph is used as fuel, along with sugars from the honey bee's stomach (Moritz et Southwick, 1992). Southwick and Heldmaier reviewed that some research have shown that a colony of bees could survive in sub-freezing temperature for a long time; a colony could retain a core temperature 31°C when the ambient temperature was -28°C, another colony has survived under the condition of -25°C for more than 300 hours (Southwick et Heldmaier, 1987).

3.1.3 Physiological changes in honey bees during winter

Honey bee colonies start brood rearing during winter with brooding peaking in spring and dropping off at the end of summer. During the warm season, late spring to summer, worker bees live briefly, living for only about 30 days. These are called summer bees. During fall, long lived bees remain in the hive. They live for up to 8 months, they are called winter bees (Döke et al., 2015). Winter bees survive during the winter by forming a heat generating cluster when the ambient temperature drops. When the brood rearing restarts, they perform a number of tasks, nursing, cleaning the cell, and foraging pollen and nectar. In this way, the honey bee colony's life cycle is continued (Döke et al., 2015).

Concerning the different physiological features between summer bees and winter bees; their Juvenile hormone (JH), vitellogenin (Vg), and the size of hypopharyngeal gland (HPG) are correlated with bee's seasonal behavior. Winter bees and nursing bees kept a low level of JH whereas, JH level in summer bees and foraging bees kept higher level. However, Vg and the size of HPG are higher and larger in nursing bees and winter bees than in summer bees (Döke et al., 2015). Among these, JH shows an important role for bees in summer, early winter, and late winter. For example, in late winter (late-January to mid-February), winter bee's JH titer begin to increase, and Vg titers, HPG size decrease like the bees those who of summer foraging bees (Fluri et al., 1982). Supporting this research, (Huang et Robinson, 1995) found that the level of JH was influenced by seasonal changes. JH biosynthesis rate has

declined from October to mid-January, and steeply increased in February. In addition, they carried out an interesting experiment to confirm the seasonal changes of JH biosynthesis and titer. This research team moved summer foraging bees into a 4°C of cold room, and their JH level remain high as would be expected. Eight days later, JH levels were significantly lower. This suggests that the factors inducing honey bee colony's overwintering state are related to temperature and endocrine secretion.

3.2 Chill coma

The topic of chill comatose in insects has received great attention from many researchers (Esch, 1988; Free et Spencer-Booth, 1960; F Goller et Esch, 1990; Hahn et Denlinger, 2007, 2011; Hosler et al., 2000; H. A. MacMillan et al., 2012; Heath A. MacMillan et Sinclair, 2011). (Free et Spencer-Booth, 1960) defined the chill coma and cold death as "the chill coma temperature is the highest temperature at which bees are immobilized by cold and the cold-death temperature the highest at which bees are killed by cold". Later (F Goller et Esch, 1990) explained as "chill coma temperatures based on ceasing of electrical activity in the flight muscles were either equal to or lower than those determined by behavioral criteria".

3.2.1 Chill coma and temperature

Earlier studies have been focused on observing chill coma temperature, chill coma behavior, and recovery time from chill coma (Free et Spencer-Booth, 1960; F Goller et Esch, 1990). Free and Spencer-Booth have determined the chill coma temperature for honey bee is 9-12°C, and occurring cold death at -2 to -6°C for group size 10 to 100 bees (Free et Spencer-Booth, 1960). (F Goller et Esch, 1990) have found similar result from 9 individual worker bees which chill coma occurs temperature at 11.2°C. this because muscles and nerves lost excitability affected by decreased ambient temperature (Esch, 1988; F Goller et Esch, 1990).

3.2.2 Muscle potential

As this thesis has mentioned above (see overwintering behavior of honey bee), when the ambient temperature drops to a certain point, bees shiver flight muscle to generate heat. (F Goller et Esch, 1990) identified 96 insects in over 20 different species (including honey bee: *Apis mellifera*) chill coma temperature, shivering behavior and muscle potential amplitudes. The muscle potential amplitude decreases with decreasing thorax temperature with an increase in duration. In honey bees, temperature drops below 20°C affected amplitudes of

muscle potential and amplitude decreases by a factor of two at 15.5°C, and finally when the temperature drops below 10°C, amplitude fell to zero. This study has figured that entering the chill coma state is linked with muscle potential in the flight muscle. Later, Hosler and colleagues found that the loss of resting potential in the muscles plays an important role of insect chill coma mechanism. According to their research, honey bees (*Apis mellifera*) and fruit flies (*D.melanogaster*) in cold conditions, muscle resting potentials decline with falling temperatures until the final burst of muscle action potentials occurs. At this stage, inward Ca^{2+} current is activated, a series of muscle action potential is generated. When it reaches the final threshold point, muscle action potential amplitude falls to zero and muscle action potentials can no longer be stimulated (Hosler et al., 2000)

3.2.3 Metabolism

With decreasing ambient temperatures, insect's energy turnover metabolism changes significantly. Briefly, oxygen consumption and ambient temperature is linearly related, oxygen consumption increases with decreasing temperature, whereas carbon dioxide release rate decreases. (Franz Goller et Esch, 1991; Kovac et al., 2007; Anton Stabentheiner et al., 2012; Auton Stabentheiner et al., 2003). Both highly active honey bees and less active honey bees show about two times higher oxygen consumption at 15°C than at 25°C. However, they could not keep up their metabolism long enough to measure ambient temperature at 10°C which is in the chill coma temperature range (9-12°C) (Auton Stabentheiner et al., 2003). In the case of CO_2, honey bee's CO_2 release rate gradually decreased with falling ambient temperature until after chill coma (below 11°C) (Kovac et al., 2007).

3.2.4 Chill coma recovery

After chill coma, when the ambient temperature is increased and normalized, insects including bees recover from chill coma, and its mechanism is reversal of the chill coma process (Free et Spencer-Booth, 1960; Jean David et al., 1998). Insect survival after chill coma is affected by several factors, exposure time, induced temperature, and acclimatization (Free et Spencer-Booth, 1960; Jean David et al., 1998; Macdonald et al., 2004). In Free and Spencer-Booth's study, honey bees who were acclimatized at 20°C woke up from comatose earlier than those who were acclimatized at 35°C. Honey bees who were exposed in chill coma for 1 hour woke up earlier than those who for 5 hours (Free et Spencer-Booth, 1960). This result was similar with other insect, fruit flies (*D.Melanogaster*) (Jean David et al., 1998; Macdonald et al., 2004). Honey bees do not appear to have adverse effects from chill coma

recovery. Studies have reported exposure to low temperature have not affected the ontogeny of foraging behavior and worker bees longevity and they were able to recover completely (Esch, 1988; Free et Spencer-Booth, 1960; Gene E. Robinson and P. Kirk Visscher, 1984).

3.2.5 Cryoprotectant

To survive and avoid physical damage from cold environment, insects have chosen an anti-freezing strategy. They accumulate glycogen reserves in their bodies and produce sugar-based molecules for cryoprotectant, such as glycerol, sorbitol, trehalose, glucose, and other sugar-alcohol(ethylene glycol, erythritol, mannitol, and ribitol) (Clark et Worland, 2008; Hahn et Denlinger, 2011; Thompson, 2003). However, honey bees have not adopted anti-freezing methods, or going into dormant stage as non-social insects. Individual honey bees cannot tolerate a severe cold condition unless they cluster and generate heat together in their colony (Free et Spencer-Booth, 1960; Southwick, 1987).

Nevertheless, unpublished results from our group found that winter bees have significantly higher levels of cryoprotectant such as sugar, trehalose, fructose, and other amino acids than those found in summer bees. This maybe suggests that winter bees could likely tolerate under chill coma condition by adapting cryoprotectant in their hemolymph as an anti-freeze. Through the research, this study hypothesized that there would be different metabolic features in between honey bees who fall into a chill coma and who show moves, do not fall into a chill coma. To further understand of honey bees, next sections (from 3.3.) will look through the colony and individual honey bee's features

3.3 What are honey bees?

Honey bees are insects, classified in the genus *Apis* and in the Family *Apidae*, as apparent in their name, honey bees collect nectar and pollen from flowers, in order to produce honey and insure the colony's survival (Winston, 1987). They are known as eusocial insects, where thousands of individuals live together forming a colony. A honey bee colony has three castes, broken up by their roles in society: queens, drones, and workers (Moritz et Southwick, 1992).

Modern honey bees include five species: *Apis dorsata, Apis laboriosa, Apis cerana, Apis florea,* and *Apis mellifera*. Among them, *Apis mellifera* is the common honey bee species worldwide. As its scientific name suggests, "honey-bearing or honey producing bee", *Apis mellifera* is a popular honey bee species for beekeeping throughout the world (Winston, 1987).

3.4 Caste and development stages

Honey bee castes can be distinguished by their sex. Female honey bees are haplodiploid, whereas males are haploid. When a queen lays an unfertilized egg, it develops into male, while a fertilized egg develops into female (Winston, 1987).

Queens Female honey bees are divided into two castes: Queens and workers. Queens tasks are few, but essential in the colony. Producing eggs is the most important duty of a queen. A fertile queen is able to lay 500-2000 eggs per day in empty cleaned cells (Hartfelder et Engels, 1998; Winston, 1987).

Anatomically, a queen has larger ovaries than the workers, and each of her two ovaries contain 150-180 ovarioles, whereas a worker has only 2-12. After queen has mated, a sperm storage organ, spermatheca, holds sperms from the drones she mates with throughout her life time, generally about 4 years. The sting of the queen is used only in battles against rival queens. It is longer than the workers and more strongly attached within the sting chamber, so that the stinger can be retracted after stinging (Snodgrass, 1925; Winston, 1987).

To control and communicate with the colony, queens produce pheromone, special chemical signals from glands. Queen mandibular pheromone (QMP): (E)-9-oxodec-2-enoic acid (9-ODA), (E)-9-hydroxydec-2-enoic acid (9-HDA), (R)-HDA, methyl p-hydroxybenzoate (HOB), 4-hydroxy-3-methoxyphenylethanol (HVA) (Slessor et al., 1988, 2005) and from several other glands: methyl oleate, coniferyl alcohol, hexadecane-1-ol, linolenic acid (Keeling et al., 2003). Those individual and blended components function to inhibit worker's ovary development, inhibit of queen rearing, attract drones for mating, stimulate comb building, and regulate worker's tasks, among other functions(Slessor et al., 2005; Winston, 1987)

Workers The workers have an enormous variety of tasks in the hive. They clean, tend to the broods, nurse larvae, feed the queen, produce wax, store nectar, collect pollen, and many other tasks which ensure the day-to-day continuity of the hive (Moritz et Southwick, 1992). Workers tasks are usually delegated by age; young workers stay in the nest, cleaning and capping the cells, and attend to the queen. Older workers perform outside jobs, such as foraging, guarding the nest, and ventilation (Winston, 1987).

Pollen is an important part of the honey bee diet, it is often called "bee bread". Worker bees hind legs (called corbicula or pollen baskets) are optimized to collect and carry pollen and propolis to the hive (Snodgrass, 1925; Winston, 1987). The workers stinger is situated in the sting chamber at the end of the abdomen (Snodgrass, 1925). It is modified for defensive

functions, and it causes worker bee's death after use, which come about within a few hours or days as a result of abdominal rupture (Winston, 1987)

Worker honey bees also produce various pheromones for communication, orientation, alarm, and defense. A study has identified 16 chemicals and others unidentified that workers produces in the pheromones (Winston, 1987). The pheromones from Nasonov gland in abdomen are the most well-known orientation odors, and are composed of seven volatile chemicals (Winston, 1987), the most active components of which are geraniol, citral and geranic acid (Moritz et Southwick, 1992). The Nasonov pheromone is important for entrance defence. The odor attracts foraging bees returning to homesite well and identifying the entrance. The Nasonov pheromone is released when a swarm is moving and orienting to the entrance of the new nest. Workers also release Nasonov pheromones for water collection (Winston, 1987). Workers produce a defense pheromone when the colony is in an emergency situation. The pattern of defensive system begins with an alert by the guard bees at the entrance (Moritz et Southwick, 1992). One of the defensive components is 2-haptanone from the mandibular glands of workers, all of other alarm pheromones are produced in the sting. The release of the alarm pheromone is related to their age, temperature, and humidity. Newly emerged workers show little response to alarm pheromones, whereas workers 5-10 days show a stronger response (Winston, 1987).

Drones The other sexual member of the colony is the drone. Drones serve a singular purpose, mating with the queen after which they die. They have large optic lobes in their brain and large eyes for finding a virgin queen, as are large flight muscle and broader wings are for flying over to the queen to mating. On the other hand, their work-related structures, such as pollen collecting structures normally found as on legs, wax producing gland, and a stinger for fighting are either reduced or absent (Winston, 1987)

Development stages (from egg to adult) All three castes of honey bees develop through four stages: egg, larva, pupa, and adult within the hives cells (Winston, 1987). After three days of embryonic development the larvae hatch from the eggs (Moritz et Southwick, 1992), after hatching, larvae begin to feed on food supplied by nurse bees (Haydak, 1970). Honey bee larvae require a lot of food for their rapid growth, all three castes gain an enormous amount of weight during the larval stage; 900, 1700, and 2300 times the egg weight for workers, queens, and drones, respectively (Winston, 1987).

A fertilized egg (female egg) can develop into a worker or a queen, depending on the rearing environment. According to Winston in the Biology of the Honey Bee, an egg laid in a worker cell can be moved into a queen cell and, under the proper conditions, will develop in to a queen; conversely, an egg transplanted from a queen to a worker cell will be reared as a

worker. Therefore, cell type and the quantity and quality of food in larval stage determines a honey bee caste, and these nutritional factors act through the larval hormonal systém (Winston, 1987).

Larvae stages are divided into five stages (L1, L2, L3, L4, L5). During the first few of days, all larvae (L1) are fed exclusively royal jelly until mid-L4, and those which are fed royal jelly after mid-L4 will develop into queens (Moritz et Southwick, 1992).

The food for the queen larvae is called "royal jelly" which is high in lipids, carbohydrates, and vitamins (Dixon et Shuel, 1963; Rembold, 1965). It is known to contain much higher proportion of mandibular gland secretion, and contains up to 10 times more pantothenic acid and 18 times more biopterin than worker jelly (Winston, 1987). During the larval stage, queens get larger quantities of food, and they are fed 10 times more often than worker larvae (Haydak, 1970). A study has found one of royal jelly proteins, royalactin, induces queen larvae development. Increases body size, ovary development, and shortens development time. Theses effects are shown, not only in honey bees, but also showed similar result in the fruit flies (*Drosophila*) (Kamakura, 2011). As a result of a different diet than the workers, queens are larger, longer-lived, have fully developed ovaries, have a sufficient quantity of ovariole, and have different adult behavior (Winston, 1987).

3.5 Age polyethism of honey bees

Worker bees perform a variety tasks during their life time. From an early age, after emergence, worker bees begin to participate in work inside the hive such as cleaning cells, rearing brood, and building combs, and outside work like guarding the colony, and foraging later in life. However, despite of all work divisions, workers spend most of the time resting and walking in the nest (Winston, 1987). Winston's "the biology of the honey bee" has refers to Lindauer's over 176 hours of observation of worker bees in hives, that worker bees were observed multiple tasks at any given age. In the observation, eighth day of worker bees showed resting, patrolling, eating pollen, cleaning cells, tending brood, and building and capping combs. Worker bees spent over two-third of their time resting or patrolling, and other third working on inside tasks (Winston, 1987). Other studies have confirmed that worker bees handle multiple tasks at each age (Seeley, 1982), and spend large amount of time patrolling and resting (Kolmes, 1985).

In spite of worker bee's resting time, a large amount of tasks are performed. Studies have listed worker's activities from 17 to 30 different tasks such as cell cleaning, capping brood, tending brood, queen tending, receiving nectar, handling pollen, comb building, cleaning

debris from the hive, ventilating, guarding the hive, flight orientation, foraging, and resting (Kolmes, 1985; Seeley, 1982). These tasks can be summarized into four groups. (1) cell cleaning and capping, (2) brood and queen tending, (3) comb building, cleaning, and food handling, and (4) outside tasks, such as ventilation, guarding, foraging (Winston, 1987).

Cleaning activities Within a few hours of emerging from their cells, bees begin their first jobs, cleaning. Cell cleaning is a responsibility for young worker bees from their emergence 0 to 5 days old (Seeley, 1982). The very earliest cell cleaning activity is removing the remains of cocoons, larval excreta, and a layer of wax from the cell. Also, cell cleaning work includes removing debris, moldy pollen, old cell cappings, and dead brood or adults. Somewhat older workers (about 11-15 days old) participate in this task, such as cleaning cell walls, smoothing the cell edges. The cell cleaning task is important, since the queen will only lay in the cell if it has been cleaned (Winston, 1987).

Brood and queen tending Brood and queen tending worker bees are known as nursing bees. Their ages vary from 5 to 13 days (Winston et Punnett, 1982) and 2 to 11 days (Seeley, 1982) respectively. According to the Winston's review, nursing bees with well-developed hypopharyngeal and mandibula glands feed brood food to larvae, and most studies indicate that the brood food glands are generally well developed by the time honey bees are 3 days old, and brood tending chores seem to peak when the bee is 6 to 16 days old (Winston, 1987). Nursing bees visit larvae cell and tend to, and feed them, and they recognize the sex and the caste of larvae (Haydak, 1958; Weaver, 1965). Feeding methods for queen larvae and worker larvae are different. Worker larvae are fed not by direct mouth to mouth food exchange, whereas queen larvae get food by direct mouth to mouth exchange from nursing bees (Winston, 1987). (Haydak, 1970) observed that a worker larva was fed 143 times in total, lasting 1 hour, 50 minutes for the whole larval period. Whereas, nursing bees visited the queen larva cell more often and longer than the worker larva cell, totaling 1600 times, lasting 17 hours. Nursing bees provide about the same amount of food to all larvae (Haydak, 1970). They recognize and quantify the larval food present in the cell. Starved larvae are fed significantly more and often (Heimken et al., 2009). A recent study has figured that the hungy larvae produce significantly high amount of the volatile pheromone E-β-ocimene, and this pheromone has attracted the nursing bee to visit to the larva cells more often. However, they did not observe more food provided to these cells. This research team assumed that honey bee larvae may use E-β-ocimene pheromone to signal their food need and attract nurses to their cell but feeding may involve other signals (He et al., 2016).

Comb building and food handling Worker bee's comb building activities usually begin at 2 days old, they age when they can produce wax, although the wax glands are most highly

developed in in workers 8-17 days old. Young age workers perform cell capping tasks, while old age workers perform comb construction (Winston, 1987). Therefore, worker bee's comb building ages vary from 2 to 20 days old (Seeley, 1982). At 20 days old, wax production in abdominal wax glands ceases (Seeley, 1989).

Worker bees receive and store nectar, and pack pollen in the cell at about the same age they begin build combs (Winston, 1987), at 11 to 20 days old (Seeley, 1982). When a worker bee receives nectar, they process the nectar into honey for storage. They receive nectar on their mouthparts, repeatedly fold and unfold their mouth repeatedly, drying out the water content in the nectar, and adding more salivary gland secretion: invertase, to cleave sucrose into fructose and glucose. After about 20 minutes, worker bees deposit the partially evaporated nectar into a honey storage cell. Fully ripened honey contains 16-20% of water (Seeley, 1995). In the case of pollen, forager bees deposit pollen in the storage cells, then the worker bees who stay in the hive take care of the rest of the food processing. They tamp the pollen down to expel the air, and then introduce a small amount of regurgitated honey, which have microbicidal effect. Through this method, they prevent bacterial spoilage and germination of the pollen. (Seeley, 1995).

Outside tasks, guarding and foraging From an age of about 20 days until their death, worker bees begin taking foraging tasks. The age of first foraging flights very variable (from 3 to 65 days old), but this activity peaks when they become about 23 days old (Winston, 1987). Foraging bees collect nectar, pollen, water, and propolis. Collecting food can differ depending on the colony's condition. A colony stops collecting nectar when they have completely filled its hives combs with honey. Similarly so in pollen collection, the colony increase pollen intake when they have a period of poor foraging. When they have abundant food, they lowering the rate of pollen collection. In case of water collection, they utilize water for cooling down the hive during hot days (Seeley, 1995).

Worker bees begin to take guarding tasks when aged 7 to 22 days. Time spent guarding varies. Most of the guarding bees only perform this tasks for less than 1 day. However, some guards continues to perform for 6 days (Moore et al., 1987). An interesting review from (Seeley, 1995) showed that some workers may never take a certain activity. Some workers who are specialized food-handling, may never guard the hive entrance, some foraging bees concentrating pollen collecting while the others focus on collecting water.

4 Materials and Methods

Study was performed at the food science department in Czech University of Life Sciences in Prauge campus, Prague, Czech Republic. Dr. Martin Kamler collected Honey bees (*Apis mellifera*) from three different hives located in the bee research institute, s.r.o., at Dol, Maslovice, Czech republic.

4.1 Sample collection and treatment

Honey bees (*Apis mellifera*) were collected on September 5th and 6th, 2018 from three different hives (Hive 181, Hive 182, Hive 183) for 20 bees each, total 60 honey bees. They were split into cages, each cage hosting bees from each hive. Each cage had a feeder containing 3:2 aqueous solution of sucrose. Cages were placed in refrigerator at 7.6°C and checked every 20 minutes for how many bees entered the chill-coma state (recognised as honey bees fund on the cage bottom in contrast to those normally crawling on the walls or sit on the feeder). The experiment was terminated at the stage when approx. 50% of the 60 bees entered the chill coma. The experiment was repeated in two independent replicates on September 5th and September 6th, 2018. In the first experiment, 50% of bees entered chill coma after 60 minutes, in the second experiment this happened after 150 minutes. After terminating, the honey bees were split into sampling vials, comatose and non-comatose from each cage separetely and samples were stored at -20°C for immediate processing.

4.2 Sample extraction for ^{1}H NMR analysis

To prepare the sample extraction, five honey bees from each group were randomly collected. Each honey bee's head was eliminated and separated gut and body. Each guts and bodies weight were measured. Honey bee guts and bodies were stored in a freezer at -20°C until proceeding sample extraction.

4.2.1 Sample grinding and extraction

Each individual frozen honey bee's degutted bodies were placed in 2mL of Eppendorf tube#1, grounded with 0.4mL of methanol in a liquid nitrogen mortar with a pre-frozen pestle. After grinding the sample, 0.8mL of methanol is added in the same Eppendorf tube.

Each Eppendorf tubes were conducted by vigorous vortexing for 15 seconds, then placed in an ultrasonic bath for 5 minutes. Samples were centrifuged at 14 000 rpm for 5 minutes. After centrifuging, 1.2mL of supernatant was transferred into a new Eppendorf

tube#2 and evaporated in a SpeedVac for 40 minutes, at 45°C. After evaporating work, added 1.2mL of methanol in the Eppendorf tube#1. Then using vortexer, untrasonic bath, and centrifuging as same as the beginning. Transferred the second supernatant (1.2mL) into the Eppendorf tube#2 and evaporating again (40 mins, 45°C).

4.2.2 Sample preparation for ^{1}H NMR analysis

1) 540µL of D_2O and 60µL of 1.5M phosphate-buffer (K_2HPO_4 and NaH_2PO_4, pH 7.40 with 5 mM TSP and 0.2% NaN_3 in D_2O) was added into the Eppendorf tube of dried sample. 2) Used vortexer for 1 minute i order to mix dried matter with solution. 3) After mixing, 600µL of sample solution was transferred in to the NMR tube (Fig.1).

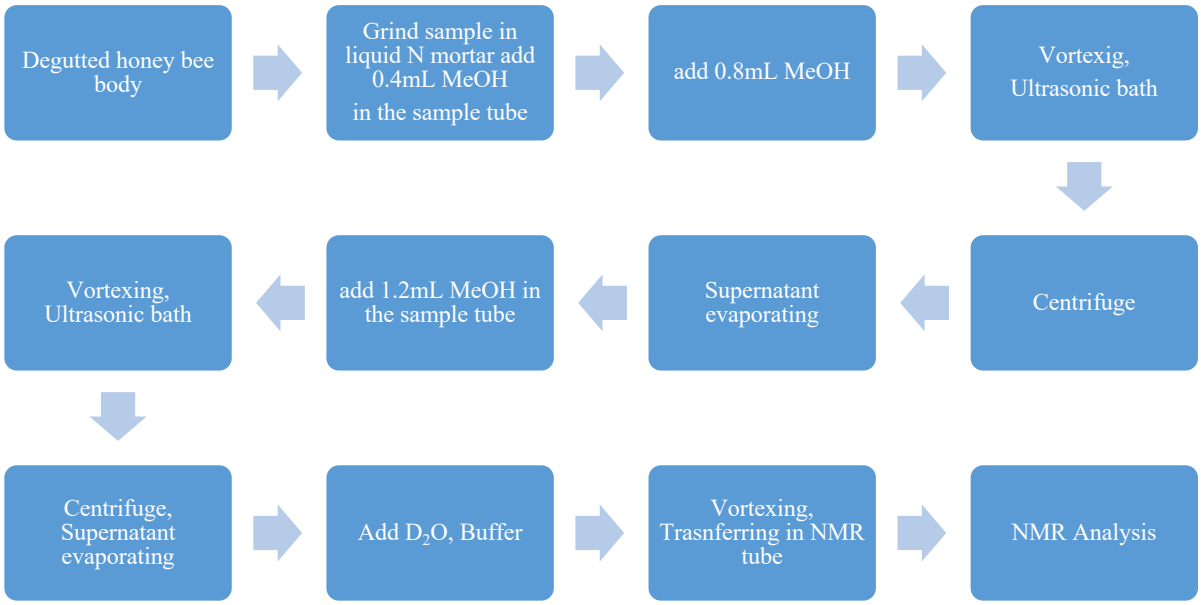


Figure.1. Sample extraction and sample preparation for NMR analysis

4.3 ^{1}H NMR measurement, data processing and analysis

The NMR measurements were performed on a Bruker Avance III (Bruker Corp., Germany), operating at a ^{1}H frequency of 500.23 MHz at 298K, 1D noesy pulse sequence, with a probe PA BBO 500S1 BBF-H-D-05 Z SP. Total 256 number of scans were acquired, spectrum width 13ppm, acquisition time was 4.9 second, mixing time 100 ms, relaxation delay of 1s and that 64k datapoint were collected. Prior acquisition, samples were locked (LOCK D2O), autotuned (ATMA), autoshimmed (TOPSHIM), set to automatic gain (RGA).

4.4 Spectra processing, non-target profiling, target profiling

The spectra were processed with the base line correction, phase correction using a software Topspin 3.5pl7 (Bruker Corp., Germany). Data binning was operated in a software MestReNova (Mestrelab research, Spain), water was removed, a single spectrum unit was 0.01ppm. Chenomx NMR Suit 8.3 (Chenomx Inc., Canada) was used for the target profiling, 45 compounds were applied for identification by Chenomx library manager and Chenomx profiling program.

4.5 Statistical analysis

To get to statistical analysis, data were applied in a statistic program MetaboAnalyst (Wishart Research group at the University of Alberta, Canada, www.metaboanalyst.ca). The data were normalized by automatically scaled. Principal Component Analysis (PCA), Orthogonal Partial Least Squares – Discriminant Analysis (OPLS-DA) were proceeded for target-profiling data. For the detail of statistical results, non-parametric test Mann-Whitney U test were applied and mean, median, standard deviation, valid N, P-value, and U-value were identified by SPSS statistics ver. 25 (IBM Corp., USA).

5 Results

The honey bee samples were marked as hive, time of exposure, and their comatose states. Eventually, we have collected 11 different groups of samples; 1) Hive 181, 60 mins, comatose, 2) Hive 182, 60 mins, comatose, 3) Hive 182, 60 mins, non-comatose, 4) Hive 183, 60 mins, comatose, 5) Hive 183, 60 mins, non-comatose, 6) Hive 181, 150 mins, comatose, 7) Hive 181, 150 mins, non-comatose, 8) Hive 182, 150 mins, comatose, 9) Hive 182, 150 mins, non-comatose, 10) Hive 183, 150 mins, comatose, 11) Hive 183, 150 mins, non-comatose (Fig.2.).

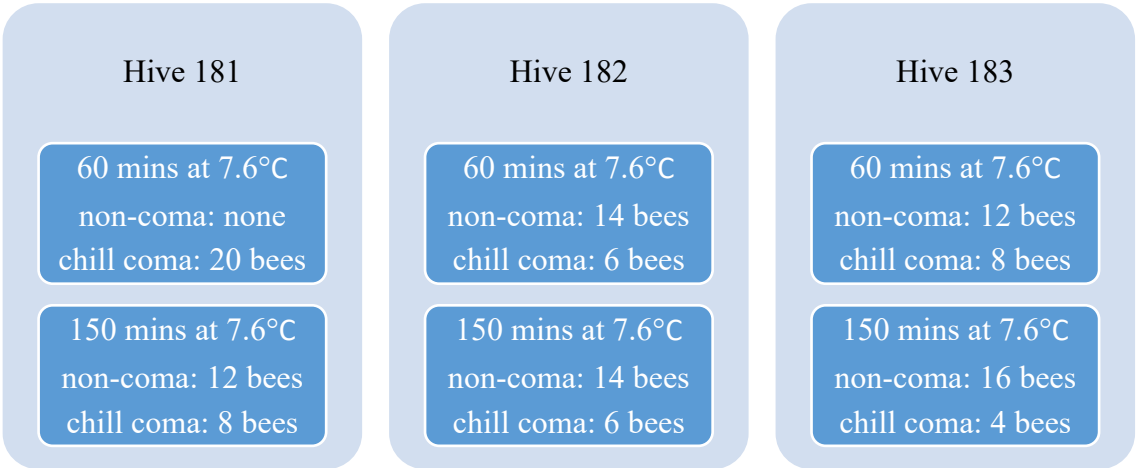


Figure.2. sample collection and division

5.1 PCA, OPLS-DA test

To find the result, this study applied PCA, OPLS-DA tests in order to define the differences between the group of honey bees which fell into chill coma and those who did not. On the PCA analysis, there were no significant divisions between the groups (Fig.3). On the OPLS-DA analysis, we could find the slight divisions between the groups and characteristic compounds (Fig.4, Fig.5). The characteristic compounds were β-alanine, acetate, glutamine, o-phosphocholine, methylguanidine, NAD+, leucine, valine, and phenylalanin (Fig.5, Table.1). However, the validity of this test was not demonstrated at 1000 permutation: Empirical p-values Q2: P = 0.006 and R2Y: p= 0.038

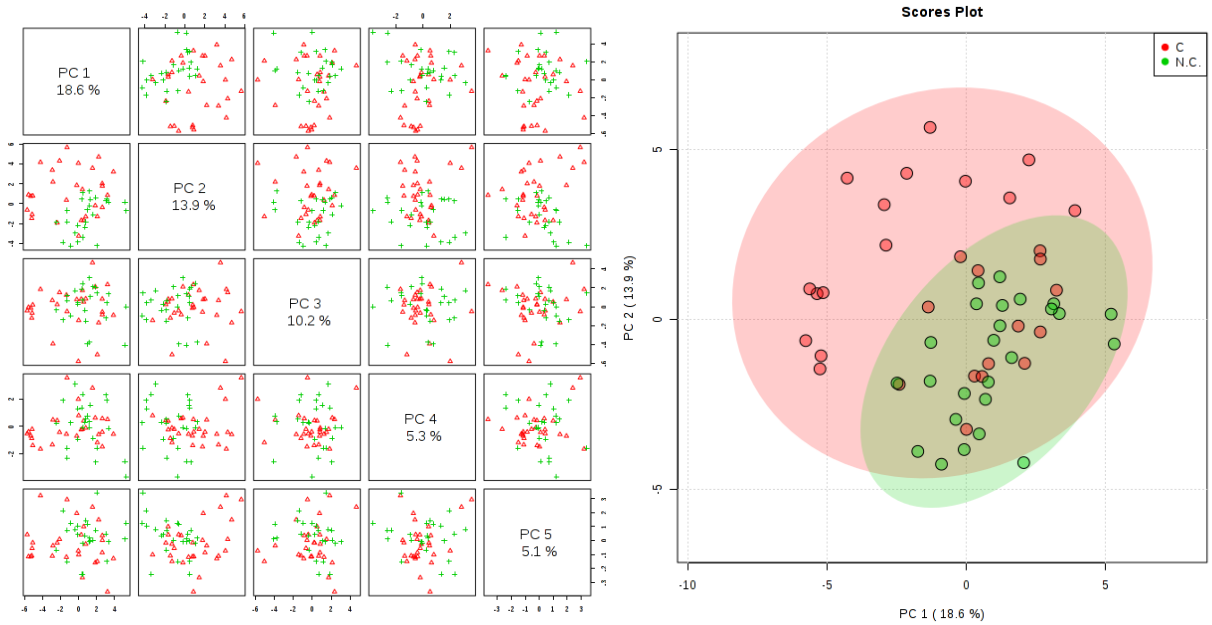


Fig.3. PCA analysis.

N.C.(green): non-coma, C (red): coma

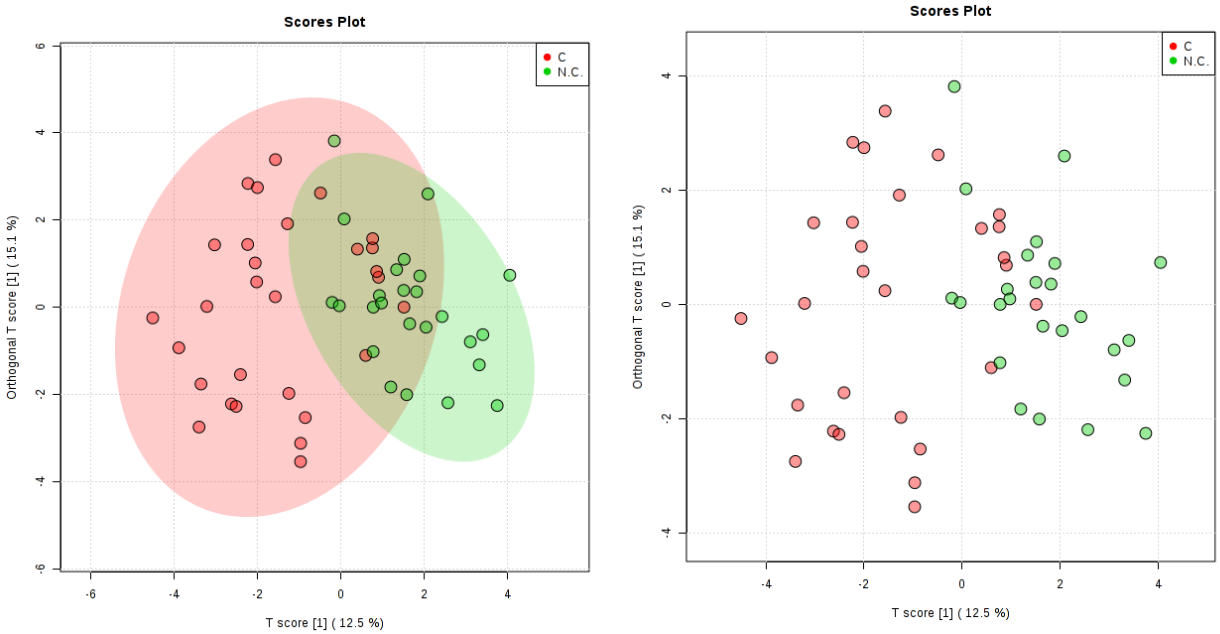
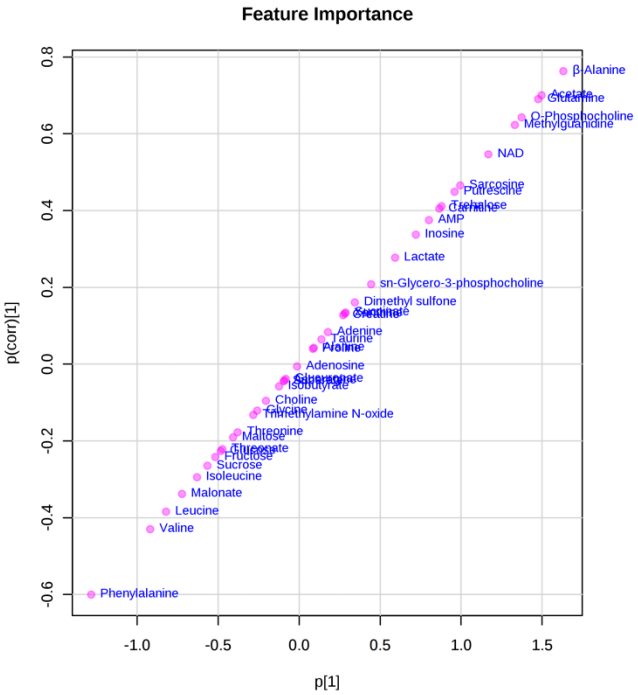


Fig.4. OPLS-DA analysis.

N.C. (green): non-coma, C(red): coma



Compounds	p(corr)
Phenylalanine	-0.60017
Valine	-0.42988
Leucine	-0.38421
Malonate	-0.33802
Isoleucine	-0.29466
Sucrose	-0.26471
NAD+	0.54638
Methylguanidine	0.62287
O-Phosphocholine	0.6425
Glutamine	0.69027
Acetate	0.70021
β-Alanine	0.76293

Figure.5. OPLS-DA analysis, significant features

Table.1. OPLS-DA feature detail table of Fig.5

5.2 Mann-Whitney U test and concentration value

This study used Chenomx program to identify and quantify compounds in the NMR spectra. A total of 45 compounds were applied for identifying, 42 were confirmed. Out of 42 identified compounds, 11 compounds showed statistically significant differences between the groups using the Mann-Whitney U test: AMP ($p = 0.021$, $N = 54$), Acetate ($p < 0.001$, $N = 54$), Carnitine ($p = 0.016$, $N = 54$), Glutamine ($p < 0.001$, $N = 54$), Methylguanidine ($p = 0.001$, $N = 54$), NAD+ ($p < 0.001$, $N = 54$), O-Phosphocholine ($p = 0.002$, $N = 54$), Phenylalanine ($p = 0.022$, $N = 54$), Putresine ($p = 0.007$, $N = 54$), Trehalose ($p = 0.024$, $N = 54$), and β-Alanine ($p = 0.001$, $N = 50$). This analysis of differences in concentration of each compounds showed how the honey bees were affected by chill coma condition (at 7.6°C).

The most abundant compounds were saccharides, fructose, glucose, sucrose, trehalose, and maltose. However, there were no statistical significant differences between the group of honey bees which fell into a comatose state and those who did not, except for trehalose (non-coma: 39.05mg/dL, coma: 25.14mg/dL, $p = 0.024$). The second highest concentration of compounds were amino acids. Among 11 amino acids, 3 amino acids showed distinct differences, glutamine, phenylalanine, and β-alanine. Glutamine concentration was 1.6 times higher in the non-coma honey bees group than the coma honey bees group (Table.2).

Interestingly, both the bees who fell in to a coma, and the bees who did not in the experiment showed a similar distribution ratio of the compound concentration (Fig.6). However, except for phenylalanine, the statistically significant 10 compounds in the non-coma honey bees group showed about 1.5 time higher on average, at minimum 1.2 times higher (putrescine) to maximum 2.4 times higher concentration (methylguanidine) than the honey bees group fell in to a comatose state (Table.2).

Among the 11 significant compounds, the most abundant compounds in the non-comatose bee group was trehalose (39.05 ± 22.83 mg/dL), o-phosphocholine (10.42 ± 2.23 mg/dL), glutamine (7.27 ± 2.74 mg/dL), β-alanine (5.57 ± 1.56 mg/dL), AMP (4.12 ± 1.34 mg/dL), aceate (2.79 ± 0.44 mg/dL), carnitine (2.46 ± 1.00 mg/dL), putrescine (1.49 ± 0.34 mg/dL), NAD+ (0.77 ± 0.24 mg/dL), phenylalanine (0.55 ± 0.45 mg/dL), and methylguanidine (0.11 ± 0.10 mg/dL) respectively, and the most abundant compounds in the comatose honey bee group were trehalose (25.14 ± 20.36 mg/dL), o-phosphocholine (8.14 ± 2.68 mg/dL), glutamine (4.33 ± 2.32 mg/dL), β-alanine (3.92 ± 1.50 mg/dL), AMP (3.19 ± 1.35 mg/dL), carnitine (1.79 ± 1.21 mg/dL), acetate (1.75 ± 0.75 mg/dL), putrescine (1.18 ± 0.38 mg/dL), phenylalanine (0.86 ± 0.57 mg/dL), NAD+ (0.51 ± 0.30 mg/dL), methylguanidine (0.04 ± 0.04 mg/dL) respectively (Table.2).

Figure.7 shows box plot images of statistically distinguished compounds which are AMP, acetate, carnitine, glutamine, methylguanidine, NAD+, o-phosphocholine, phenylalanine, puterescine, trehalose, and β-alanine.

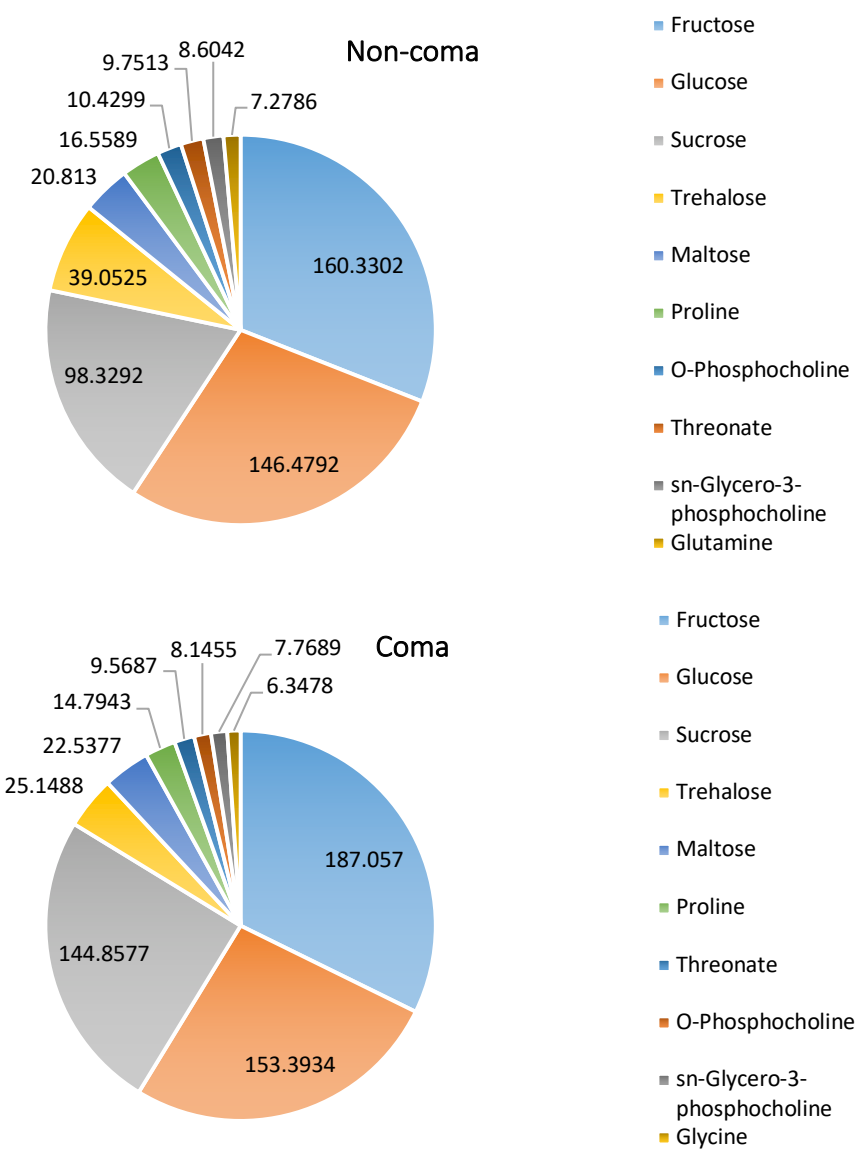
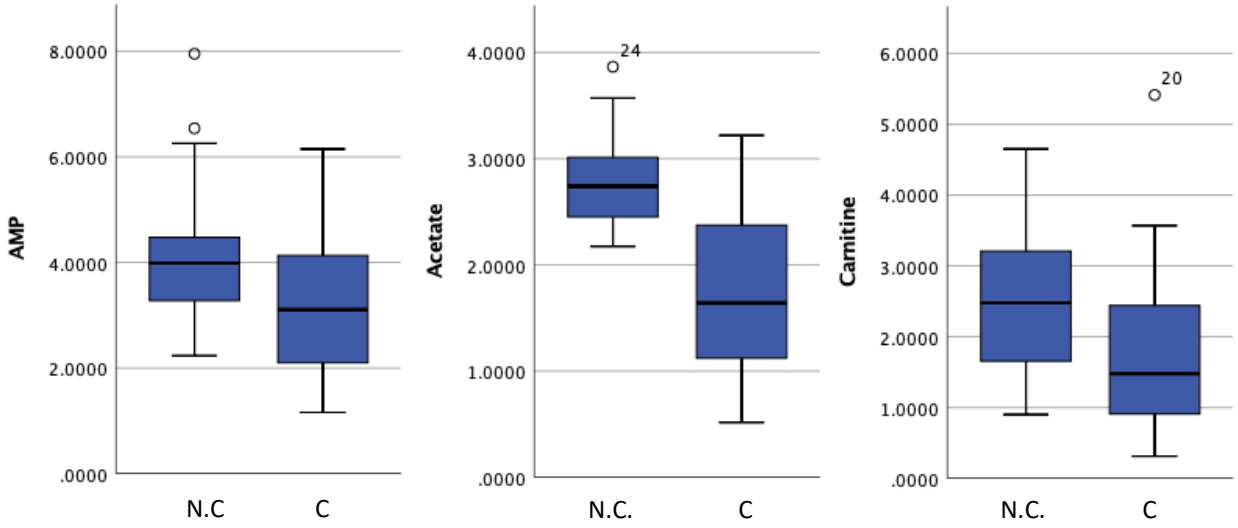
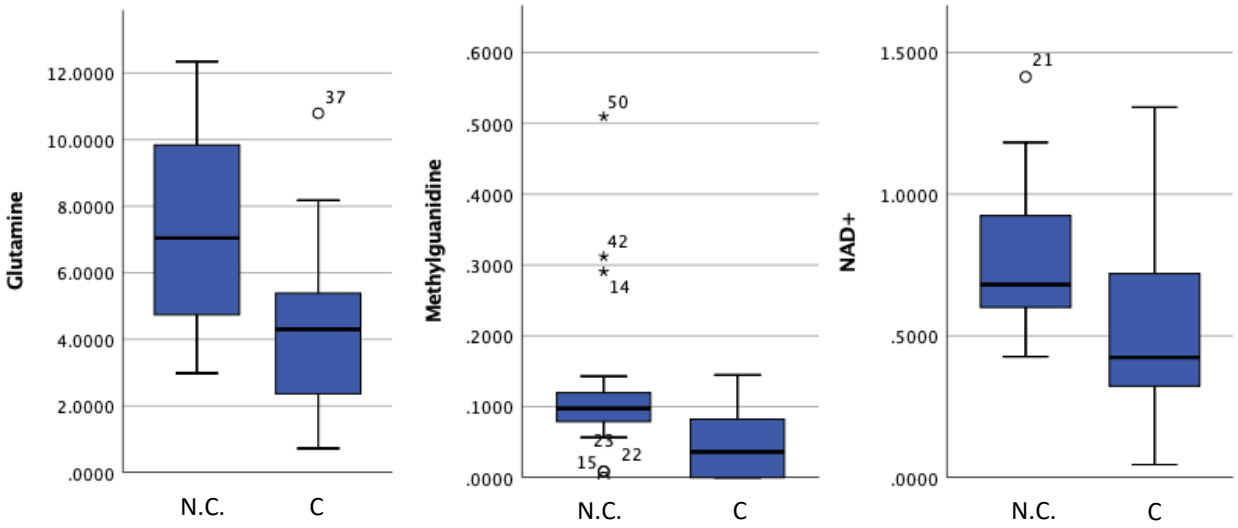
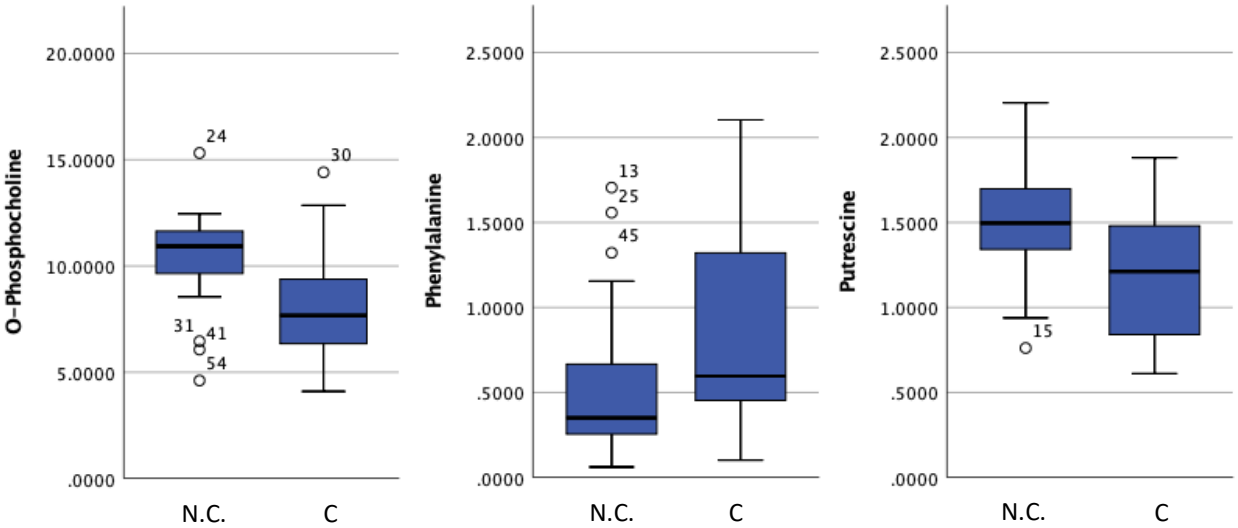


Fig.6. Similar concentration (mean value) ratio of the non-coma and the coma group


AMP
8.0000
6.0000
4.0000
2.0000
.0000
N.C
C
Acetate
4.0000
3.0000
2.0000
1.0000
.0000
24
N.C.
C
Carnitine
6.0000
5.0000
4.0000
3.0000
2.0000
1.0000
.0000
20
N.C.
C


Glutamine
12.0000
10.0000
8.0000
6.0000
4.0000
2.0000
.0000
37
N.C.
C
Methylguanidine
.6000
.5000
.4000
.3000
.2000
.1000
.0000
50
42
14
25
22
15
N.C.
C
NAD+
1.5000
1.0000
.5000
.0000
21
N.C.
C


O-Phosphocholine
20.0000
15.0000
10.0000
5.0000
.0000
24
30
31
41
54
N.C.
C
Phenylalanine
2.5000
2.0000
1.5000
1.0000
.5000
.0000
13
25
45
N.C.
C
Putrescine
2.5000
2.0000
1.5000
1.0000
.5000
.0000
15
N.C.
C

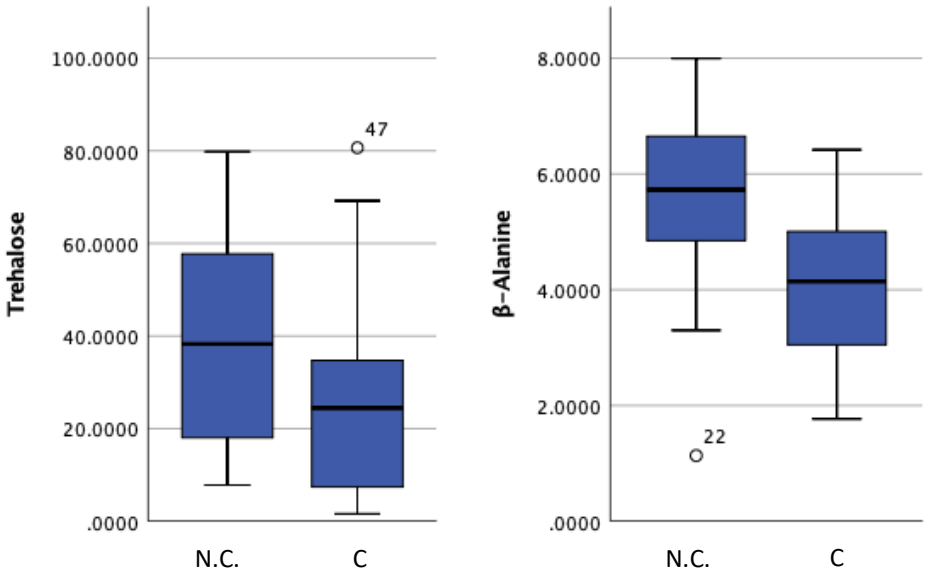


Fig.7. Concentrations of the compounds that were significantly different (P<0.05) between comatose and non-comatose bees (X axis: N.C means non-coma, C means coma, Y axis: concentration)

Table.2. Statistics for individual compounds

	Non-coma				Coma					
Compound	**Mean**	**S.D.**	**Median**	**Valid N**	**Mean**	**S.D.**	**Median**	**Valid N**	**P value**	**U value**
AMP	**4.1252**	**1.3417**	**3.9864**	**25**	**3.1959**	**1.3517**	**3.107**	**29**	**0.021**	**229**
Acetate	**2.7926**	**0.4414**	**2.741**	**25**	**1.7545**	**0.7563**	**1.6426**	**29**	**0**	**93**
Adenine	0.1743	0.0738	0.1943	5	0.1417	0.0868	0.1197	5	0.548	9.5
Adenosine	0.2674	0.2045	0.1979	5	0.2366	0.0821	0.2417	8	0.833	22
Alanine	6.6063	3.1889	5.978	25	5.3363	2.7582	4.8794	29	0.109	270
Asparagine	1.2888	0.4231	1.4289	9	1.5854	1.1821	1.1831	9	0.931	42
Carnitine	**2.4626**	**1.0057**	**2.479**	**25**	**1.7936**	**1.2137**	**1.478**	**29**	**0.016**	**224**
Choline	4.2401	1.2314	4.0637	25	4.0582	2.1753	3.1456	29	0.163	282
Creatine	0.5328	0.206	0.5691	25	0.4795	0.2013	0.4416	29	0.353	309
Dimethyl sulfone	0.1047	0.1034	0.0797	18	0.0861	0.05	0.0794	20	0.874	174
Fructose	160.33	180.899	52.0125	25	187.057	243.111	76.0129	29	0.993	362
Glucose	146.479	129.376	67.3177	25	153.393	178.078	81.6244	29	0.573	330
Glucuronate	2.7036	0.977	2.9598	4	2.8305	2.1647	2.6253	5	0.905	9
Glutamine	**7.2786**	**2.743**	**7.0415**	**25**	**4.338**	**2.3293**	**4.2987**	**29**	**0**	**152**
Glycine	6.9865	7.9351	3.2111	25	6.3478	6.7771	3.5601	29	0.527	326
Inosine	3.8911	1.2389	4.0636	18	3.4653	1.0886	3.5197	16	0.281	112
Isobutyrate	0.0701	0.0262	0.0832	5	0.0894	0.0927	0.042	5	0.841	11
Isoleucine	1.5044	0.5748	1.3656	25	1.4662	0.7775	1.4495	29	0.538	327
Lactate	0.9814	0.6918	0.9814	2	0.2989	0.078	0.3371	3	0.2	0
Leucine	1.3302	0.5413	1.2756	25	1.6381	0.9039	1.4893	29	0.362	415
Lysine	-	-	-	0	0.505	-	0.505	1	-	-
Malonate	0.242	0.168	0.1665	16	0.3964	0.2384	0.4385	20	0.062	219
Maltose	20.813	35.3658	2.5614	15	22.5377	31.2193	9.2263	22	0.249	203
Methylguanidine	**0.119**	**0.1073**	**0.0975**	**25**	**0.0485**	**0.0469**	**0.0362**	**29**	**0.001**	**176**
NAD+	**0.7754**	**0.248**	**0.6804**	**25**	**0.5152**	**0.3057**	**0.4235**	**29**	**0**	**156**
O-Phosphocholine	**10.4299**	**2.2375**	**10.9278**	**25**	**8.1455**	**2.6873**	**7.6768**	**29**	**0.002**	**180**
Phenylalanine	**0.5505**	**0.4536**	**0.3505**	**25**	**0.8602**	**0.5767**	**0.5964**	**29**	**0.022**	**495**
Proline	16.5589	6.165	16.8747	25	14.7943	6.5488	14.9881	29	0.294	302
Putrescine	**1.4961**	**0.3435**	**1.4961**	**25**	**1.1885**	**0.3821**	**1.2116**	**29**	**0.007**	**208**
Sarcosine	1.2851	0.7332	1.1026	25	0.9361	0.4934	0.8298	29	0.057	253
Suberate	0.3791	0.2438	0.2942	4	1.0927	0.0643	1.0927	2	0.133	8
Succinate	0.581	0.2584	0.509	25	0.4846	0.261	0.4283	29	0.091	265
Sucrose	98.3292	197.597	16.8842	25	144.858	201.496	35.5955	29	0.4	411
Taurine	6.7974	-	6.7974	1	3.401	-	3.401	1	1	0
Threonate	9.7513	8.0097	6.861	5	9.5687	8.9118	5.3762	10	0.859	23
Threonine	1.3407	0.4679	1.5584	3	1.3646	0.7136	1.0295	5	1	8
Trehalose	**39.0525**	**22.8327**	**38.3022**	**25**	**25.1488**	**20.3685**	**24.4662**	**29**	**0.024**	**232**

Trimethylamine N-oxide	1.2488	0.652	1.2031	25	1.3344	0.6601	1.4168	29	0.621	391
Valine	1.501	0.701	1.5342	25	1.9755	1.2651	1.5285	29	0.286	424
Xanthine	1.3552	0.469	1.4618	25	1.15	0.6675	1.1717	29	0.263	298
sn-Glycero-3 -phosphocholine	8.6042	2.8788	8.68	25	7.7689	3.1114	7.2704	29	0.319	305
β-Alanine	**5.5768**	**1.5666**	**5.7281**	**25**	**3.925**	**1.5036**	**4.1416**	**25**	**0.001**	**134**

Concentration Mean, Standard Deviation, Median, Valid N, P-value, and U-value of Non-coma, Coma state of Honey bees. Unit: mg/dL

6 Discussion

This study found that the most significant metabolic features between honey bees who did not fall into a chill coma and those who fell into a chill coma were the detected levels of AMP, acetate, carnitine, glutamine, methylguanidine, NAD+, o-phosphocholine, phenylalanine, putrescine, trehalose, and β-alanine.

The highest concentration in the honey bees group which showed non-coma was trehalose, also the non-coma honey bees have 1.5 times higher concentration of this compounds than in the honey bees group which fell into a chill coma. Trehalose is present significantly high concentrations in insect blood and it constitutes the major haemolymph (blood) sugar. Trehalose in the insect is also called “the insect blood sugar”. Glucose and fructose are also present, but generally lower (Becker et al., 1996; Thompson, 2003; Woodring et al., 1993).

In cold conditions, honey bees begin shivering flight muscles to create heat energy (See. Overwintering section). When an insect powers their flight muscle, glycogen in the muscle and the haemolymph disaccharide, trehalose are oxidized and used for energy by the pathways of glycolysis and TCA cycle. The metabolic breakdown leads the resulting heat production (Moritz et Southwick, 1992; K. B. Storey, 1985; Thompson, 2003). A study looking at blood sugar levels in honey bees showed interesting results, that a honey bee’s blood sugar level changes after an hour of cold exposure. The study measured the bee’s fructose, glucose, and trehalose levels and showed that the levels of fructose and glucose after cold exposure decreased subtly whereas trehalose levels decreased by 1.7x after cold exposure. Throughout this study, I had assumed that honey bees mainly used trehalose for cold tolorence while exposed in the low temperature (See Table.3) (Woodring et al., 1993). Therefore, I suggest that the higher concentration of trehalose in non-coma honey bees shows that this compound was essential for cold tolerence in the honey bees of this thesis.

The increased rate of trehalose in insect haemolymph during cold exposure has been confirmed by several studies. (Kimura et al., 1992) found that trehalose content in four *Drosophila* species were gradually increased during cold seasons mid-October to December. In the other study, trehalose was higher in cold acclimated insects, two different wheat weevils (Canadian strains of *S. granarius*, French strains of *S. granarius*) and beetles (*C. ferrugineus*). Also, the insect (Canadian strains of *S. granaries*) who showed stronger cold hardiness than the others had higher trehalose level than the other insects (Fields et al., 1998). Common fruit fly (*D. melanogaster*) showed a similar result as well. (Overgaard et al., 2007) used NMR spectroscopy to examine the insect metabolite changes in a rapid cold hardening

treatment (25°C to 0°C at -0.1°C/min and held at 0°C for 1 hour) and a cold shock treatment (-5°C for 1 hour). The study found that trehalose levels were elevated significantly after the both rapid hardening treatment and cold shock treatment. However, a study of flesh flies (*S.crassipalpis*) found different results that the trehalose level was significantly reduced in cold exposure (at 4°C for 8 hours) compared with the normal condition at 25°C (Michaud et Denlinger, 2007).

Table. 3. Initial sugar titers of blood sugars and the effect after cold exposure in honey bees

Condition	**Initial blood sugar (mg/ml)**			**After 60 mins (mg/ml)**		
	Fructose	Glucose	Trehalose	Fructose	Glucose	Trehalose
Cooled bees	4.3±0.9	5.2±0.3	17.6±1.9	3.0±0.3	3.1±0.3	9.9±1.6

Cooled bees were quickly cooled at 0°C by immersion in chipped ice 5 times in 60 mins. This table is adopted and modified by the Woodring and colleagues Table 1. (Woodring et al., 1993)

High levels of certain metabolite concentrations can be indicated by a broad range of metabolic process. However, it can be a good indicator of physiological processes of adaptation. Sugars, in a role as cryoproectant is a good example. As I have mentioned above (See. 3.2.5. Cryoprotectant), many insects accumulate sugars such as glycerol, trehalose, glucose, and various proteins in their haemolymph and tissues to avoid freezing and to survive low temperatures (Thompson, 2003). (R. E. Lee, 1991) reviewed over 20 insects' freeze tolerance and their cryoprotectatns. The most common cryoprotectant was glycerol, followed by other polyols, and trehalose. However, this thesis experiment did not apply to examine glycerol or other polyol sugars in the honey bees, and the cooling temperature was enough to put the honey bees into chill coma (honey bee chill coma range: 9 to 12°C, experienced at 7.6°C) but it was not enough to see anti-freezing activation. This suggests that the next study wll find more detail of cryoprotectant compounds such as glycerol, sorbitol, and other polyol sugars in honey bees, in several different conditions.

Cold stress in insects is associated with the level of increasing amino acids (Lalouette et al., 2007; Overgaard et al., 2007; K. Storey, 1983). Proline is used as an energy fuel by many insect species, and both proline and alanine in flight muscle and fat body are metabolically related to generate energy (Lalouette et al., 2007). According to Storey's explanation, "two oxidative reactions convert proline to glutamate which then enters the TCA cycle at α-ketoglutarate after transamination. When used as the flight fuel the proline is

conversion to alanine. Alanine is returned to the fat body where in combination with acetyl-CoA proline is resynthesized" (K. B. Storey, 1985). In both common fruit flies (*D.melanogaster*) and beetles (*A.diaperinus*) an increasing level of alanine and a decreasing level of proline was shown after cold exposure (Lalouette et al., 2007; Overgaard et al., 2007). Therefore, we expected a meaningful rate of proline or alanine in both honey bee groups. However, neither proline nor alanine showed significant differences between the non-coma honey bee group and the coma honey bee group.

The other amino acid, glutamine, can be broken down to release energy via the TCA cycle intermediates. As a consequence of amino acid oxidation, ammonia or ammonium is produced as a by-product. Ammonia can be assimilated by glutamate (Glu) to yield glutamine (Gln) which was found in high concentrations in cold-exposed beetles (Lalouette et al., 2007), common fruit flies (Overgaard et al., 2007), and flesh flies (Michaud et Denlinger, 2007). As well as in this thesis' experiment in the non-coma group of honey bees.

Lipids, fatty acids are efficient energy fuels. Insects store lipids mostly as triglycerides in the fat body and supplied into the hemolymph as diacylglycerol which is transported into the tissues. Several studies reviewed insect lipid utilization (Arrese et al., 2001; Arrese et Soulages, 2010; K. B. Storey, 1985). Fatty acids are oxidized in mitochondria by the β-oxidation pathway to convert into acetyl-CoA molecules which for further oxidation and energy production by TCA cycle. For this oxidation to occur, carnitine is responsible for transferring fatty acids to the mitochondria matrix from the cytosol (Pratt et Cornely, 2018). In this thesis, carnitine showed a significant concentration in the non-coma honey bees group rather than in the coma honey bees group. We can assume that high concentration of carnitine in non-coma honey bees group could support fatty acids convert into energy to generate heat by using flight muscles.

Age could be one of the factors of honey bees chill coma responses. A study found that honey bees in different ages showed different reactions in cold temperature, where old bees (age 15 to 28 days) had a lower chill coma temperature thresholds than young bees (age 1 to 14 days). This can be explained that young bees have a lower metabolic rate than older bees (Free et Spencer-Booth, 1960). Similar results were shown by (Auton Stabentheiner et al., 2003), comparing older honey bees and younger honey bees oxygen consumption during falling ambient temperature. Both older bees and younger bees' oxygen consumption rates increased during lower temperatures, but older bees showed higher oxygen consumption than the younger bees. Thus, ages should be considered in honey bee's chill coma responses.

7 Conclusions

Through the experiments, we were able to find the honey bee's chill coma responses and metabolic differences from cold exposure. Using the ^{1}H NMR spectroscopy, we could examine a total of 11 significant metabolic compounds which may play important roles for a honey bee's cold tolerance. This could give us to a better understanding as to how the individual's physiology affects the honey bee colony through out winter.

Honey bees are by far the best studied eusocial insects (Winston, 1987), but studies about the honey bee's chill coma reaction and consequences of metabolism are relatively understueid compared to many other insects. From the surveyd literature, we could find that our study had similar results with other insects's cold exposure reaction which is meaningful. However, we should consider that each insect species metabolism reactions and the crucial compounds could be different. Thus, furthrer detailed studies are recommended to understand honey bee's chill coma metabolism.

8 References

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