ANALYSIS OF ENVIRONMENTAL CUES CAUSING THE SEASONAL CHANGE IN PGM (PHOSPHOGLUCOMUTASE) ALLOZYME FREQUENCIES IN HONEYBEES (*APIS MELLIFERA* L.)

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I hereby declare that all information in this document has been obtained and presented in accordance with academic rules and ethical conduct. I also declare that, as required by these rules and conduct, I have fully cited and referenced all material and results that are not original to this work.

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ABSTRACT

ANALYSIS OF ENVIRONMENTAL CUES CAUSING THE SEASONAL CHANGE IN PGM (PHOSPHOGLUCOMUTASE) ALLOZYME FREQUENCIES IN HONEYBEES (*APIS MELLIFERA* L.)

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In an earlier project completed in our laboratory a seasonal fluctuation in Phosphoglucomutase (PGM) phenotype frequencies was found, so that the winter bees were almost all heterozygotes and long lived than the summer bees among which homozygotes were significantly at high frequencies at *Pgm* locus. Same results were obtained in populations of three subspecies, *A. m. meda*, *A. m. caucasica*, and *A. m. carnica* from different climatic regions. In the current study environmental cues related with seasonal change in PGM phenotype frequency was examined along with the correlation between PGM heterozygosity and overwintering success. Cessation of food influx was found to be effective by itself as an environmental cue that causes a sudden and sharp

increase in PGM heterozygosity. In addition to that, PGM heterozygosity of the colonies with greater overwintering success was found to be significantly higher than the ones with intermediate or low overwintering success.

Benefiting from the previous studies and the results of current study, ethyl oleate was suggested as a chemical signal that functions in the regulation of PGM heterozygosity.

Keywords: Honey bee, *Apis mellifera*, phosphoglucomutase, PGM, heterozygosity, allozyme frequency, environmental cues.

ÖZ

BALARILARINDA (*APIS MELLIFERA* L.) PGM (FOSFOGLUKOMUTAZ) ALLOZİM SIKLIKLARINDA MEVSİMSEL DEĞİŞİME NEDEN OLAN ÇEVRESEL TETİKLEYİCİLERİN İNCELENMESİ

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Laboratuarımızda daha önceden gerçekleştirilmiş bir projede Fosfoglukomutaz (PGM) fenotip sıklıklarında, *Pgm* lokusunda kış arılarının neredeyse tamamının heterozigot olduğu ve homozigotların kayda değer ölçüde yüksek sıklık gösterdiği yaz arılarına kıyasla daha uzun ömürlü olduğu mevsimsel bir dalgalanmanın varlığı gözlemlenmiştir. Değişik iklim bölgelerine ait üç alttür olan *A. m. meda, A. m. caucasica* ve *A. m. carnica* toplumlarında aynı sonuçlara ulaşılmıştır. Bu çalışmada, PGM fenotip sıklığı ile ilişkili çevresel tetikleyiciler yanında PGM heterozigotluğu ile kışlama başarısı arasındaki ilişki incelenmiştir. Yiyecek akışının kesilmesinin PGM heterozigotluğunda ani ve çarpıcı bir artışa neden olan bir çevresel tetikleyici olduğu bulunmuştur.

Bunun yanında, daha yüksek kışlama başarısına sahip kovanların PGM heterozigotluğu orta ve düşük kışlama başarısına sahip kovanlarınkinden kayda değer biçimde yüksek bulunmuştur.

Daha once gerçekleştirilen çalışmaların ve bu çalışmanın sonuçlarından yararlanılarak etil oleat, PGM heterozigotluğunun düzenlenmesinde rol oynayan bir kimyasal olarak ileri sürülmüştür.

Anahtar kelimeler: Balarısı, *Apis mellifera*, fozfoglukomutaz, Pgm, allozim sıklığı, çevresel tetikleyiciler

To my beloved Deniz

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Let us honor the memory of thousands of bees sacrificed for the sake of this thesis.

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LIST OF ABBREVIATIONS

PGM : Phosphoglucomutase

HK : Hexokinase

G-1-P : Glucose-1-Phosphate

G-6-P : Glucose-6-Phosphate

G-6-PDH : Glucose-6-Phosphate Dehydrogenase

$MgCl_2$: Magnesium chloride

NADP : Nicotine amide adenine dinucleotide phosphate

Mg : Magnesium

PMS : Phenasine metosulphate

Tris : Tris [Hydroxymethyl]-aminomethane

HCl : Hydrochloric acid

ATP : Adenosine triphosphate

MTT : 3-(4,5-Dimethyl-2-Thiazyl)-2,5-Diphenyl-2H-Tetrazolium Bromide

CHAPTER 1

INTRODUCTION

1.1. Biology of the Honey Bees

Honey bees are social insects that form complex communities with socially regulated division of labor in a similar manner with eusocial ants and wasps (Schulz *et al*., 2002). Bees that form such social communities are classified under the Apinae subfamily of Apidae family. Apinae subfamily includes four species of honey bees: *Apis dorsata*, *Apis florae*, *Apis cerana* and *Apis mellifera* L., the Western honey bee (Engel, 1999).

Honey bees are important organisms not only ecologically buy also economically. They obtain such importance by the activities they have to go through with in order to maintain their survival. As the bees forage for nectar and pollen they simultaneously contribute to the pollination of plants. The value of crop pollination by the honey bee is estimated to be 5 to 14 billion dollars per year in the United States alone (Kremen *et al*., 2002). Being an important pollinator of countless ecosystems of the world, bees are in a mutualistic interaction with flowering plants that frequently leads to co-evolution (Kiester *et al*., 1984).

1.2. Colony Members and Social Life

Honey bee colonies vary in size from 10000 individuals in winter to 70000 in summer (Van Nerum & Buelens, 1997). Bee society is divided into three

castes: queen, the only reproductive female; workers, non-reproductive daughters of queen which develop from fertilized eggs; and drones, male progeny of the queen which develop from unfertilized eggs and has a single role in the colony i.e. insemination of the queen. Caste is basically trophic and is independent of genetic differences (Brian, 1957).

Sustainability of the social structure of a colony is maintained through complex interactions of individuals in the hive (Robinson, 1999). Alarm substances and cast pheromones have been defined since 1960s (Wilson, 1965). With the emergence of advanced biochemical techniques, scientists were able to gather information on the production and transfer of pheromones (Naumann *et al.*, 1991; Naumann *et al*., 1993), and their effects on hormones of the honey bee (Kaatz *et al.*, 1992). Further investigation of the honey bee pheromones have shown that each individual in the colony is capable of releasing pheromones and in turn chemical communication in the hive is multidirectional and highly complex (Leoncini *et al.*, 2004; Slessor *et al.*, 2005). However, visual signals and touching between the members of society are also very efficient ways that bees use to communicate (Kroeber, 1952).

Food sources of honey bees can be divided into two: Abundant food of the colony is honey and nectar which are rich in carbohydrates; and pollen, which is the principle protein source of the colony, is rather limited (Schmickl & Crailsheim, 2002). Being the limited principle source of protein, pollen available per larva has a significant effect on the amount of brood, the brood rearing efficiency and age of first foraging. It has been shown that when the pollen storage is deficient, nursing activities focus on older larvae and the younger larvae are neglected (Schmickl & Crailsheim, 2002; Crailsheim *et al.*,

2003). When the nutrition is limited, honey bee colonies respond by adjusting their physiology (Mattila & Otis, 2007) and demography (Willard *et al.*, 2011) Workers have other roles besides nursing the larvae. Polyethism in honey bee is a socially regulated and age dependent process in which individuals pass through behavioral transitions over time so that their task in the colony is determined in an age related fashion (Allan *et al*., 1987; Page & Peng, 2001). Maturation of bees from eggs into winged adults takes about 21 days during which the organism goes through egg, larval and pupal stages. Cleaning the empty cells for egg laying is a task of newly emerged bees. 3-14 day old bees are able to produce wax to prepare and repair cells as well as synthesize jelly, food for the larvae and the queen. These workers are called nurse bees (Toth & Robinson, 2005). When they are 15-20 day old building combs, processing nectar, guarding the colony against intruders, and transporting the dead bees out of the hive becomes duties of the worker bees. At the end of second or third week of their lives workers start foraging for resources outside the hive such as nectar, pollen, and water. Foraging requires flight which has a high energy demand (Huang & Robinson, 1992). All the activities that worker bees perform in or out the hive are crucial and they need to be done simultaneously for the survival of the colony. Simultaneous performance of these diverse duties requires physiologically and behaviorally differentiated worker bees to exist in the colony at the same time which is obtained by the age related division of labor (Schulz *et al*., 2002). However, neither physiological nor behavioral changes of the aging bee are on a fixed schedule; they are highly flexible (Schulz *et al.*, 2002) and responsive to the social (Robinson, 1999) and physical (Willard *et al.*, 2011) cues coming from the environment of the bee. Moreover, not each individual of the colony are equally responsive to the signals such as pheromones of the hive (Pankiw *et al.*, 1994). Behavioral transitions in the colony can not only be delayed or accelerated but also be

reversed depending on the level of pressure generated by the needs of the colony (Robinson, 1992; Pettis *et al*., 1995). Despite many animal taxa go through behavioral transitions only once as they enter a new environment or life history stage in adulthood, adult behavioral development in honey bees is profoundly plastic and responsive to environmental factors including social influence (Roberts & Elekonich, 2005). It has been shown that when there is a shortage of nurses in the hive, bees delay their transition to outside activities and keep feeding the larvae at their older age (Robinson *et al.*, 1989). The opposite of this phenomenon is also observed, where precocious foraging is observed in the absence of foragers in the colony (Huang & Robinson, 1992). It has been demonstrated in various studies that the genetic background of the colony (Giray & Robinson, 1994) and environmental factors such as season (Huang & Robinson, 1995; Robinson *et al.*, 1992) and nutritional status (Willard *et al.*, 2011) effect adult behavioral development of honey bees. The physiological changes become apparent as the bees age and transfer to working outside the hive; an increase in the juvenile hormone levels, water content of the body, glycogen content of the thorax, metabolic rate and flight capacity and a decrease in body mass is observed (Huang *et al*., 1994; Roberts & Elekonich, 2005). Activities of various enzymes in different tissues have been reported to alter as the age dependent behavioral development of the bee progresses (Roberts & Elekonich, 2005). The question how seasonal changes in the age demography of the colony and metabolism, physiology and activities of the bees are associated with enzyme loci seems to be a significant one.

1.3. Honey Bees in Winter and Summer

Honey bees exist in a wide range of climatic regions because of their geographical distribution and they have to bear with temperatures much below

freezing point and higher than 40^{o}C depending on the season and the climate (Heinrich & Esch, 1994). Honey bee colonies in summer and winter are different for their behavior, physiology, population structure and age demography (Bodenheimer, 1937; Seeley & Visscher, 1985).

The change of seasons from autumn to winter is accompanied with a series of change in the colony which turns the population structure from an active brood-filled summer colony to an hibernating broodless winter colony (Mattila & Otis, 2007). This dramatic transition observed in honey bee colonies has to be directed by the changing environmental factors. As a matter of fact along with photoperiod and temperature, diminishing pollen sources was shown to have significant effect on timing of both the beginning of the decrease in brood-rearing activity and the increase in the longevity of workers (Mattila & Otis, 2007). Colonies with greater pollen storages were observed to produce a greater number of workers into the autumn with regard to colonies with lesser pollen supplies (Mattila & Otis, 2007).

A summer colony consists of up to 70000 bees with a life span around 30 days (Van Nerum & Buelens, 1997). Whereas, a winter colony consists of 10000 to 20000 individuals with a life span up to several months (Mattila & Otis, 2007). Longer life span of the winter bees is of critical value for the survival of the colony through the winter since only very little brood can be reared during that time (Mattila & Otis, 2007; Van Dooremalen *et al*., 2012). Another interesting phenomenon about the bees is that they have to endure not only limited food and water resources, and low temperatures but also hypoxia through the winter. It was shown that under low oxygen conditions bees lower their metabolic rate and go into dormancy which can be reversed once the oxygen level is restored (Van Nerum & Buelens, 1997).

Even though winter bees do not perform foraging flights as summer bees, they have a high energy demanding job to do: producing heat to stabilize the temperature in the hive. Clusters are formed by winter bees with a core temperature up to 40°C and a surface temperature of 12°C (Stabentheiner, 2003). Winter cluster is generally formed by bees when the temperature falls to 15°C (Kronenberg & Heller, 1982). Bees contract their thoracic muscles to produce heat and keep the core area of the cluster at such high temperatures despite the environmental temperature and the bees on the surface of the cluster provide insulation (Esch & Bastian, 1968). Bees in the cluster breakdown carbohydrates for thermoregulation by the release of heat energy from their flight muscles (Stabentheiner *et al*., 2002). Energy demands of thermoregulation and flight were found to be similar as well as metabolic state and glycogen stores of heating bees (bees in cluster) and the foragers (Crailsheim & Panzenböck, 1997). Moreover, glycogen content of honey bees varies depending on their position in the cluster. Glycogen reserves of the surface bees are significantly higher than glycogen reserves of the bees in the core of the cluster (Crailsheim & Panzenböck, 1997). Infrared inspection of the clusters has shown increased body temperature of the bees at the core region of the cluster which indicates increased metabolic rate for those bees and hence increased consumption of stored glycogen which in turn can explain the gradual change in the glycogen content from the core to the surface (Crailsheim & Panzenböck, 1997). A similar trend in glycogen reserves was shown when summer and winter bees were compared. Glycogen reserves of winter bees were shown to be higher than that of the summer bees (Crailsheim & Panzenböck, 1997). Seasonal variation as well as the variable levels of glycogen storage among the layers of winter cluster points to the contribution of glycogen reserve to the social thermoregulation which is a unique adaptation

honey bees have evolved to survive through the winters (Crailsheim & Panzenböck, 1997).

1.4. Polymorphism and Basic Principles of Isozymes

Polymorphism is defined as simultaneous occurrence within or between populations of multiple phenotypic forms of a trait attributable to the alleles of a single gene or the homologs of a single chromosome (Acquaah, 1992). Recurrent mutations in the genes of individuals cause variation in natural populations. However, loci may be polymorphic or monomorphic (Zeidler, 2000). Metabolic pathways are composed of many enzymes that function in harmony in which each isozyme has its specific role. It is not rare for isozymes to be tissue or cell specific (Markert, 1975). Enzyme polymorphism provides flexibility, versatility, and precision, in other words phenotypic plasticity, in the metabolic functions of an organism and thus, molecular variation increases the fitness of organisms through its positive effect on metabolic efficiency (Zeidler, 2000).

The mechanisms by which isozymes are formed can be covered in two main groups, i.e. genetic and epigenetic. Genetic mechanisms include gene duplication through mutation, polyploidization and chromosomal aberrations (Zeidler, 2000). Four genetic systems have been suggested explaining the genetic make-up of the observed enzyme polymorphisms (Acquaah, 1992). These are; multilocus system I where independent proteins with the same enzymatic activity are coded by different genes, multilocus system II where different subunits of a polymeric enzyme are coded by multiple loci, multilocus-polymeric system where enzymes display a series of polymers that

consist of identical subunits, and allozyme system where alleles of a single locus code for the variants of the enzyme (Zeidler, 2000).

Epigenetic mechanisms can be listed as post-translational addition, post-translational deletion, and post-translational conformation.

1.5. Use of Isozymes and Allozymes as Genetic Markers

Allozymes are defined as different allelic forms of a given enzyme coding gene, whereas isozymes are defined as different forms of functionally similar enzymes encoded by different loci (Markert & Møller, 1959; Lewontin & Hubby, 1966; Prakash *et al*., 1969). The importance of isozymes for population genetics was not appreciated until within population polymorphism for isozymes was discovered (Stebbins, 1989; Wendel, 1989). Once the significance of isozyme polymorphism as a tool for population genetics was understood it started to be used in a wide range of species for that purpose such as aspen species (Liu & Furnier, 1993); microsporidian, *Nosema grylii* (Dolgikh *et al.*, 1997); *Drosophila spp*. (Parker *et al.*, 1998, Buth & Murphy, 1999); and broadtail shortfin squid, *Illex coindetii* (Martínez *et al.*, 2005) besides hybrid populations of many plant species (Cruzan, 1998) Since the primary products of the genes are the peptides they code rather than the ultimate observable phenotype which can be the result of the combined effect of multiple genetic and environmental elements. With the introduction of gel electrophoresis of proteins population genetics was able to make precise quantitative estimates of genetic variability. While the data from gel electrophoresis had been compiling, genetic distance or similarity coefficients were developed summarizing the allozyme data which provided the means of comparisons of intra and inter-population genetic variations (Avise, 1974).

Genetic structure of a population can be assessed from allozyme data with allele and phenotype frequencies used to estimate heterogeneity level, gene diversity, and gene flow. Once those parameters are obtained, deviations from Hardy-Weinberg equilibrium and linkage disequilibrium can be used to infer the situation of a population (Futuyma, 2004).

Allozyme studies have been carried out for about 50 years which provided a large amount of comparable data and application of numerous standard statistical methods gave information on the genetic variation in populations. It is convenient to use allozyme instead of nucleic acid variation since allozymes mostly have codominant loci distributed according to Mendelian laws of inheritance and most of them are expressed at all developmental stages of the organism (Hamrick, 1989).

1.6. Adaptive Consequences of Enzyme Polymorphism

Foundation of the neutral theory of molecular evolution was inevitably followed with the neutralist-selectionist argument on the adaptive value of enzyme polymorphisms (Kimura, 1968). According to the neutral theory of molecular evolution, majority of polymorphisms at molecular level are selectively neutral and random fixation of selectively neutral or nearly neutral mutations but not Darwinian selection is the predominant cause of evolutionary changes at molecular level (Kimura, 1986). However Kimura stated that it should be clarified that neutral theory of molecular evolution does not completely exclude natural selection as a mean of adaptive evolution but rather claims that most of the changes in DNA do not bear any adaptive value.

Neutral theory was supported by theory of metabolic flux which claims that

because of the complexity of the regulatory networks and great number of loci interacting in the metabolic pathways, the effect of polymorphism in a single enzyme would be minor (Kacser & Burns, 1981). Supporters of the theory of metabolic flux also note that *in vitro* conditions are not adequate in imitating *in vivo* conditions. Nevertheless, there are examples of *in vivo* measurements of enzyme activity that are consistent with *in vitro* measurements in the literature. For instance, activity measurements of 6-phosphogluconate dehydrogenase (6-PGD) and glucose-6-phosphate dehydrogenase (G6PD) allozymes *in vivo* and *in vitro* are congruent (Cavener & Clegg, 1981).

There was opposition to the claim that enzyme loci themselves were not being selected even in the very early days of isozyme research (Markert, 1968). Opponents argued that selection could be particularly effective on the enzymes positioned at regulatory points of metabolic pathways (McDonald, 1983; Watt *et al*., 1985) and via altering the metabolic flux, these key point enzymes may have an effect on overall fitness of the organism (Johnson, 1973; Karl & Avise, 1992). Later on the idea that enzyme polymorphisms at the branching points of pathways may be more prone to selection pressures and have a greater influence on the metabolic flux was further supported (Eanes *et al*., 2006; Verrelli & Eanes, 2001a).

There are many examples of how variation in a particular enzyme can affect the fitness of the organism. Investigation of polymorphism in Phosphoglucomutase (*Pgm*) locus of *Drosophila melanogaster* has revealed that different phenotypes exhibited different catalytic capacity and PGM activity and glycogen content are positively correlated (Verrelli & Eanes, 2001b). *Pgm* locus was shown to have the highest polymorphism among all enzyme loci that play a role in glycolysis and Citric Acid Cycle in *Alvinella*

pompejena, a polychaete worm (Jollivet & Desbruyeres, 1995). A study of 17 metabolic enzymes in *Drosophila* has revealed that only 5 of them were not consistent with neutral evolution and 4 out of those 5 enzymes were G6P branch point enzymes including PGM each with a significant role in flux control (Flowers *et al*., 2007). In another study it was shown that different PGM phenotypes have different thermostability profiles which has an effect on the distribution range of polychaete worm (Piccino *et al*., 2004).

In a study on dung flies (*Scathophaga stercoraria*), a seasonal variation of PGM phenotypes was shown with an increased frequency of the most common allele in the spring and autumn compared to the summer (Ward, *et al*., 2004). In the conclusion of the same study interesting study seasonal distribution of individual dung flies (*Scathophaga stercoraria*) at the mating site was explained by a selection model in which mortality of different PGM phenotypes are determined by the differential effect of temperature on the flight capacity (Ward *et al*., 2004). The adaptive role of allozymes in a marine snail, *Littorina saxatilis*, was studied across the vertical rocky-shore gradient and the greatest differentiation between ecotypes was observed for *Pgm-2* (Galindo *et al*., 2009). High level of differentiation for *Pgm-2* was explained by selective advantage of genotypes $Pgm\text{-}2^{110/100}$ and $Pgm\text{-}2^{110/110}$, which are thermostable even at temperatures up to 35°C, over the genotype $Pgm\text{-}2^{100/100}$ which exhibited an important loss of function at high temperatures; as a result, providing adaptation to high temperature across the vertical environmental gradient (Galindo *et al*., 2009).

Gpi is yet another locus that was found to have a positive effect on the Darwinian fitness in of sea anemone, *Metridium senile* through modulation of carbohydrate metabolism at glycolysis-pentose shunt branch via its allozymes

as the environmental temperature fluctuates (Hoffmann, 1981). Different *Pgi* allozymes in leaf beetle, *Chrysomela aeneicollis* were found to have a significant effect on fitness of larvae and thermal behavior of adults (McMillan *et al*., 2005).

A common assumption of allozyme polymorphism levels being temporarily constant has caused underestimation of the adaptive value of changing allozyme frequencies with the transition of seasons (Moffett & Crozier, 1996). Seasonal alterations in allozyme frequencies were explained by differential gene expression as a response to temperature (Podrabsky, 2004; Somero, 2010). The mechanism of this response may be more related to epigenetic effects rather than genetic variation at the enzyme loci (Allendorf *et al*., 1983).

In nature, it is odd to find an environment that stay same for large periods of time and thus, in the course of evolution species have obtained the ability to sustain variation in their morphological, behavioral, and biochemical properties which itself is an adaptive trait that increases the fitness of organisms in a changing environment. Kinetically favored phenotypes of PGI and PGM were found to have advantage under certain environmental conditions in Colias butterflies (Watt *et al.*, 1985). Wu & Seliskar (1998) found that an alternative form of plasma membrane H^+-ATPase in *Spartina patens* is related to salinity adaptation in that salt marsh plant species. A link between heat shock protein expression and PGI phenotype for natural populations of montane insect species, *Chrysomela aeneicollis* was demonstrated in a study by Dahlhoff & Rank (2000). In another study on *Chrysomela aeneicollis* by the same authors, different PGI phenotypes were suggested to have different effects on both HSP expression and cold tolerance which gives the species an advantage in adapting itself to the climate change

(Rank & Dahlhoff, 2002). The correlation between PGI phenotypes of *Chrysomela aeneicolli* and extreme weather conditions was shown by other authors as well and PGI enzyme was suggested to be under temperature selection in beetle larvae, and adults (McMillan *et al.*, 2005). In that frame, enzyme polymorphism can be seen as a mechanism which gave the organisms a flexible metabolism that can react with the changes in the environment in an adaptive manner (Johnson, 1973).

1.7. PGM Enzyme and Its Role in Energy Metabolism

Carbohydrate metabolism is defined as biochemical processes responsible for the formation, breakdown and interconversion of carbohydrates in organisms (Polerstock *et al*., 2002). Carbohydrates are metabolized easier than fats and proteins. which makes them the most important energy source of honey bees (Crailsheim & Panzenböck, 1997). Since honey bee has a specialized carbohydrate-rich diet and nutrient dependent caste determination, carbohydrate-metabolizing enzymes are important in the honey bee (Kunieda *et al*., 2006).

There are mainly six metabolic pathways concerning carbohydrate metabolism: glycolysis, carbon fixation, pentose phosphate pathway, glycogenesis, gluconeogenesis and glycogenolysis (Scrutton & Utter, 1968).

In glycolysis energy is provided by converting glucose into pyruvate which is then oxidized forming acetyl CoA that enters the Citric Acid Cycle for further breakdown followed with the aerobic oxidation through the respiratory chain to yield energy (Voet & Voet, 2011). Glycolysis is a very well conserved and universal metabolic pathway descended since very ancient times of life on

earth (Hochachka & Somero, 2002). The glycolytic pathway starts with hexokinase catalyzed reaction phosphorylating the 6^{th} carbon on glucose and by doing so enabling metabolisation of glucose. Once phosphorylated, glucose has three probable fates; continue through glycolysis to produce energy, enter the glycogenesis to be converted into glycogen for storage, or enter pentose phosphate pathway to be used in anabolic processes such as formation of amino acids and nucleic acids (Voet & Voet, 2011).

In muscles glycolysis takes place not only to produce ATP in the absence of oxygen but also provide a rapid source of ATP in order to make macroscopic motion possible. Since Citric Acid Cycle and the respiratory chain are not involved in the ATP production, the steady state is reached much faster in glycolysis (Easterby, 1981). The amount of glucose required to produce same amount of ATP with aerobic metabolism is approximately 17 folds higher. Thus, for the muscles to be able to produce enough energy via glycolysis there needs to be an excessive glucose stored in the vicinity of contractile myofibrils (Meléndez-Hevia *et al*., 1993).

Phosphoglucomutase catalyzes the reversible transfer of a phosphate group between glucose-1-phosphate and glucose-6- phosphate, the first intermediate in glycolysis. Glucose-6-phosphate can now theoretically travel down the glycolysis or pentose phosphate pathway. Thus, PGM is a key enzyme in energy metabolism functioning at a branch point of the glycolytic pathway dividing into the glycogen synthesis, the pentose shunt, and the main glycolytic corridor (Carter & Watt, 1988). In other words PGM plays an important role in directing and regulating the metabolic flux of energy metabolism in all cells and functions in both glycolysis and gluconeogenesis. Figure 1 shows a simplified scheme of glycogen synthesis and degradation

where PGM is labeled with a red frame around it.

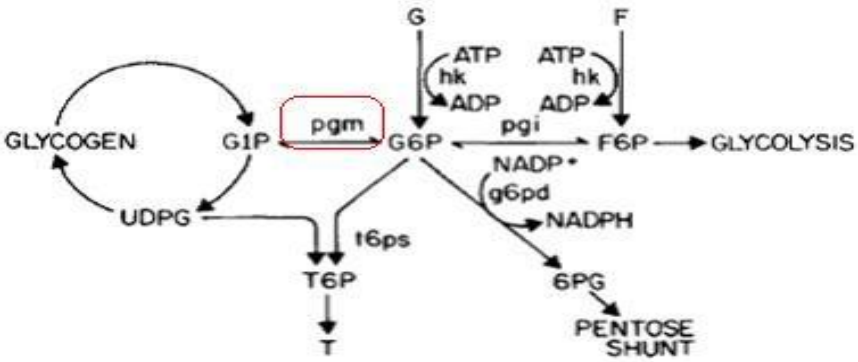

Figure 1. Simplified scheme of glycogen synthesis and degradation (Carter & Watt, 1988). Abbreviations: ADP, ATP; adenosine di- and triphosphate, F; fructose, G; glucose, 1-P or 6-P; -1- or -6-phosphate, g6pd; glucose-6-phosphate dehydrogenase, hk, hexokinase, pgi; phosphoglucose isomerase; pgm, phosphoglucomutase; NADP and NADPH**,** oxidized and reduced forms of nicotinamide adenine dinucleotide phosphate, 6PG**,** 6-phosphogluconate, t6ps; trehalose-6-phosphate synthetase.

1.8. Seasonal Change in PGM Heterozygosity

It has been shown that PGM in honey bees have two common alleles that can be differentiated on starch gel electrophoresis; the fast allele, Pgm^{100} and the slow allele Pgm^{75} (Kandemir *et al*., 2000). Heterozygosity in PGM has been used in research on population genetics of honey bee along with many other enzymes (Nunamaker & Wilson, 1981; Robinson & Page, 1989; Sheppard &

Berlocher, 1989). Many of those studies have shown a correlation between PGM heterozygosity and climatic region (Lobo *et al.*, 1989; Lama *et al.*, 1990; Bouga *et al.*, 2011). In two successive studies conducted in our laboratory it has been shown that PGM heterozygosity is not a fixed value for a population of honey bees over time (Kence, *et al.* 2006). When the same hives were sampled for each month of the year it was clearly seen that regardless of the subspecies, climatic region, or specific population of bees being sampled, heterozygosity is always less than 10% in summer bees as opposed to more than 90% in winter bees (Güldüren, 2007). Another important finding on PGM heterozygosity was that even though summer bees are highly homozygous, heterozygosity of forager bees in summer months were as high as that of worker bees in winter. Moreover, spectrophotometric analyses of PGM variants have revealed that heterozygotes have significantly higher enzymatic activity and glycogen content (Güldüren, 2007). The common selective pressure on worker bees of winter and forager bees of summer is the extremely high energy demand of flight muscles which are excessively used for long distance flights during which foragers may carry nectar and pollen that weighs up to 80% of their own body weight, and maintaining the temperature of winter cluster under hypoxic conditions of the hive. Foragers in summer and the whole colony in winter have to be able keep up with this extreme energy demand or else the survival of the colony would be endangered. So being able to store large amounts of glycogen in the muscles as a fuel for extreme levels of glycolysis during flight or heating has a selective advantage and is exactly what was observed in foragers and winter worker bees (Güldüren, *et al.* 2008).

Another case where PGM heterozygosity was found changing with the season is in Clauded sulphur butterflies (*Colias philodice eriphyle*) and exposure for selection due to acute demand by flight metabolism and nectar limitation was

proposed as an explanation for the phenomenon (Carter & Watt, 1988).

1.9. Objective of the Study

Main objectives of this study are:

To test the effect of two environmental cues for seasonal change, temperature and foraging based food, on PGM enzyme polymorphism.

To understand the timing of the effect of environmental cues, i.e. whether the cue has an effect before eggs laid or during later development in terms of expression of specific allozymes, on the observed PGM polymorphism. Enzyme electrophoresis will be used in order to examine allozyme frequencies in both larvae and adult worker bee samples from hives exposed to environmental manipulations.

To investigate if there is a correlation between PGM phenotype frequencies and overwintering success

CHAPTER 2

MATERIALS & METHODS

2.1. Samples Used for Allozyme Analysis

In total 1016 worker honeybees were studied in the experiments realized for this thesis. Queens were not used for obvious result it would bring, i.e. loss of the whole colony, and drones were not used due to their haploid genome. Samples were collected and brought alive to the laboratory. Then they were labeled and kept at -80^{o}C until they were used for gel electrophoresis.

2.2. Chemicals Used in the Analyses

All chemicals were purchased from SIGMA chemical company, US. A list of the chemicals used in all analyses can be seen in Appendix A.

2.3. Sampling

In order to investigate a possible correlation between overwintering success and PGM heterozygosity, at the beginning of spring when the temperature got above 15^{o}C and flight started (03.19.2012), "strength" of colonies were assessed according to the ratio of survivors at the end of winter to the population size in the beginning of the winter. Colonies were classified as strong, intermediate, and weak with survivor ratios of more than 80%, around 50%, and less than 30%, respectively. Adult worker honey bees were sampled

from inside the hive on 03.19.2012 and 03.26.2012.

Manipulation experiments were carried out in order to test the effects of temperature, and cessation of food influx on PGM heterozygosity starting on 08.08.2012 and ending on 10.12.2012. Those dates were chosen since according to the previous results (Kence *et al., 2008*) the lowest PGM heterozygosity level is observed at the end of July and thus probable effects of manipulations would be detected. A hive was taken from the apiary and placed into a cold room in which the temperature was fixed at 4^{o}C, another one was left at its original location with an artificial rain system dripping water to the entrance to keep the honey bees from leaving the hive, and the third hive was used as a control without any manipulations. Initially adult worker honey bees were sampled from inside the hive to note the "zero" state of each colony before any manipulations. Another sampling was done at the end of 4^{th} week on 09.04.2012. Also newly laid eggs were located and marked for follow up. An exchange of eggs between cold and control hives was done to test whether the environmental conditions are affecting the queen bee so that "different" eggs are laid or the individuals themselves are affected as they develop. Last sampling was done a week later on 09.12.2012 during which larvae developed from previously marked eggs in the "rain" colony were collected as well. None of the eggs transferred from or into the "cold" hive have developed into larvae. In the 36 days long time interval of manipulations weather conditions were fairly stable with day temperatures around 30^{o}C and night temperatures around 15^{o}C with only one cloudy and four rainy days.

Later on a new "cold" hive and control hive were chosen to repeat the egg exchange experiment; however, once again eggs failed to develop into larvae. A new couple of hives were chosen as "cold" hive and control hive, and this

time the cold room temperature was adjusted to 10^{o}C on 09.27.2012 and adult worker bees from inside the hive were sampled. Eggs were observed in both hives on 10.01.2012, and egg exchange between the hives was done. Milder conditions did improve the success of the experiment; except the ones that were laid in the control hive and transferred into the cold hive, all eggs developed into larvae. Larvae and adult worker bees were sampled one week later.

2.4. Electrophoretic Analysis

2.4.1. Preparation of the Homogenates

Previous studies have almost always advised to separate the thoraces of adult bees from their abdomens and discard the abdomens, proceeding the homogenization with only thoraces. However, initial experimentation of starch gel electrophoresis of samples with or without abdomens had not manifested any differences in the resolution, precision, and the general pattern of the PGM bands observed. Using Tris-EDTA Buffer or distilled water as a homogenization solvent also did not cause any differences. As a result we chose the simplest method which is homogenization of complete adult worker bees without any dissection in 500µl distilled water per sample. Larvae were homogenized in 50µl distilled water since the small samples as they are could easily be over diluted causing an unwanted loss of visibility of PGM bands on the starch gel. The homogenates were kept in ice until centrifugation in a pre-cooled machine at 4^{o}C and 10000g for 10 minutes in order to preserve the PGM activity as much as it could be which is necessary in the staining step. Supernatants were transferred into microtubes and stored in -80°C until used.

2.4.2. Preparation of the Starch Gel

Electrophoresis in general requires an electric field, a buffer system and a medium for the samples to move through. Samples are loaded to various media by various methods and a tremendous variety of buffer systems are used for the conduction of electricity. Once the samples are loaded and the current is sustained, electric field creates a force on the samples as long as they carry some electrical charge on them, which is a very common property of biological molecules. The distance that a molecule can migrate on the electrophoresis medium at a given time depends on the of the strength of the electric field applied, temperature, concentration and the nature of electrophoresis medium, and the charge, size and conformation of the molecule moving on the electric field. Since the variables related with the electrophoresis will be the same for all of the molecules loaded on the same system, the principle cause of distinction of molecules on the electrophoresis is the differences in their charge, size, and conformation.

Thin layer native horizontal starch gel electrophoresis was employed in this study. The basic equipment for electrophoresis is illustrated schematically in Figure 2.

The quality of the material used has a profound effect on the quality of the starch gel prepared. Brand of starch, the quality of distilled water, starch lot, laboratory and refrigerator temperatures, and the length of time that gels are vacuum-degassed are some of the factors that need to be considered as effectors of gel quality (Conkle *et al*., 1982). In preliminary studies 8% was found to be the optimal concentration for the starch gel electrophoresis of PGM. In the later experiments we had to order a new stock of starch from

SIGMA since the old stock was about to end. Even though exactly same product from exactly same manufacturer was used, starch concentration had to be increased to 11% to obtain same level of polymerization with this new lot of starch.

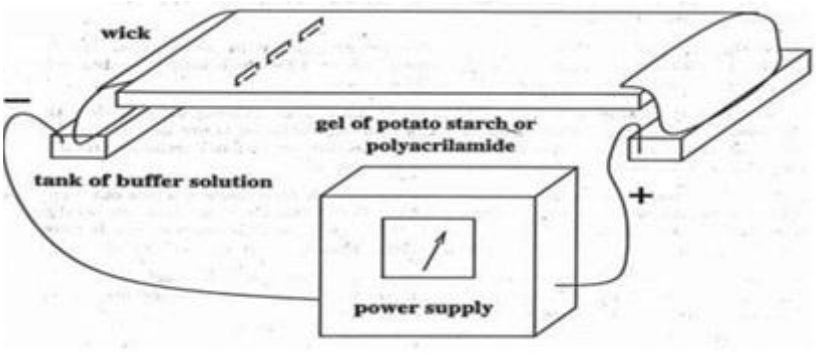

Figure 2. Illustration of the horizontal starch gel electrophoresis equipment.

2.4.3. Procedure

Electrophoretic assays were optimized from the methods used in Shaw and Prasad (1970) and McDonald (1985) and carried out on a Multiphor II Electrophoresis Unit. (Amersham Co. US).

8% or 11% starch gel solutions with a total volume of 170 ml (V_T=152 ml distilled water and 17 ml buffer) were prepared in a flask. The gel was heated on a Bunsen burner with continuous vigorous stirring to avoid sticking to the bottom of the flask and burning, or clumping until it finally thickens and starts

to boil. Then the gel was degassed with a vacuum pump for 15-20 seconds until small bubbles disappear and large bubbles (water vapor) appear. After degassing, the gel was poured into the gel mold on a glass plate confined with two plexiglass frames on top of each other with inner dimensions of 10cm x 20cm and a height of 3mm each. Air bubbles were picked out if there were any.

The mold was let cool at room temperature for about half an hour (the gel is now a semi-transparent white color which was initially almost fully transparent with a hint of yellow).Then, the gel mold was put into the refrigerator for about an hour to accelerate polymerization. After the refrigeration, the gel is almost fully opaque with a profound white color. The fully polymerized gel is cut through the frames horizontally into two with the help of a thin copper wire and the upper half of the gel is flipped over on another glass plate so that smooth surfaces of both sides are facing upwards. The gels are ready to load.

The gels are placed on the cooling plates of Multiphor II Electrophoresis Unit for as soon as they are ready to load. Loading process is performed on these cooling plates and all the homogenates are kept in ice for the whole time of loading. Whatman no 3 filter paper is cut into rectangular pieces with dimensions about 3mm x 6mm to produce wicks. Wicks are dipped into the supernatant obtained from centrifugation of homogenates and checked for excess loading which can be inferred from shiny/wet appearance of the wicks; excess sample is eliminated by gently touching the wick to a clean piece of tissue paper. Wicks are stabbed into the starch gel to form two straight lines that are about 5cm apart and extend from one long side of the gel to the other. A paper wick is dipped into Bromophenol Blue solution and loaded to the end

of the line of samples that is closer to the negative electrode to observe the effect of the current. About 300 ml of +4 ^{0}C electrode buffer (given in Table 1) is poured into electrode trays and the clothes saturated with buffer are placed on both ends of the gel and extended down into the tray to contact electrode buffer. Then the voltage and the current of the power supply are turned on. The cooling unit of the electrophoresis tank maintained at 4°C throughout the run. The electrophoretic system and running conditions for PGM are given in Table 1.

Table 1. Electrophoretic conditions for PGM.

Enzyme	Buffer System	Running Temperature	Voltage & Current	Running Time	References
PGM	Tris-Maleate pH. 7.4	4 °C	170 V 30mA	5 hrs.	McDonald, 1985 Shaw&Prasad, 1970

2.4.4. Staining

Once predetermined running time of the gel for the separation of allozymes has passed, the gel is removed from electrophoresis system for staining. Histochemical staining (Soltis *et al.*, 1983) is applied combined with agar overlay method (Whitmore, 1990) in which a solution of enzyme-specific substrate and cofactor, and oxidized salt along with a dye that precipitates on

the site of enzyme catalyzed reaction is mixed with a cooled agar solution and poured onto the gel as a second layer. Then, the gel needs to be kept in a dark incubator at 37°C. Since stains containing PMS (Phenazine metosulphate) and MTT [3-(4,5-Dimethyl-2-th,azolyl)-2,5-diphenyl-2H-tetrazolium bromide] turn blue after 15-20 minutes in the light, a dark incubator is essential and 37°C temperature is chosen as it is the optimum for enzymatic reactions. In this method it is not directly the physical presence of the enzyme as a protein but rather the activity of that enzyme that is being detected in an indirect manner. That is why this method is also called "Activity Staining" and the researcher has to conserve the activity of the enzyme starting from sampling, through the steps of homogenization and electrophoresis until the staining has successfully completed. If applied successfully, dark blue bands start to appear on the gel in 15-30 minutes and get darker as incubation continues. The stain ingredients for PGM are given in Table 2. The detection principle in activity staining can be summarized as below:

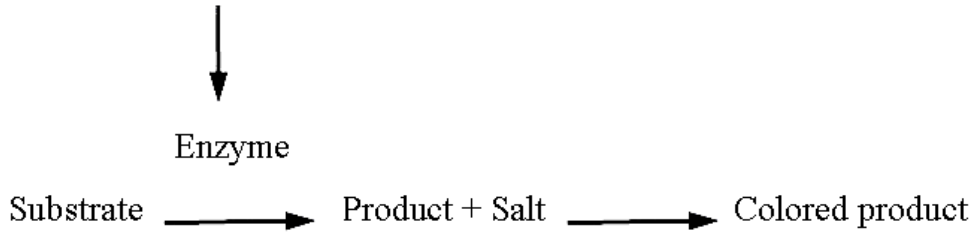

Table 2. Staining ingredients for PGM.

Enzyme	Stain Ingredients (for 100ml	Amount used
PGM	Tris-HCl pH. 8.0 staining buffer	50ml
	1.5% Agar Solution	50ml
	Glucose-1 -Phosphate	50mg
	$MgCl_2$	50mg
	NADP	10mg
	MTT	10mg
	PMS	3mg
	Glucose-6-Phosphate Dehydrogenase	30units

2.4.5. Interpretation

The length, charge and conformation of the enzyme are taken into consideration while interpreting the gel results. Changes in the primary structure - i.e. the amino acid sequence - of a protein alter the net charge which can be detected on the gel since relative mobility varies. There are basic assumptions when the starch gel results are being interpreted. If an enzyme is monomeric, i.e. it only has a single polypeptide chain, homozygotes have a single band and heterozygotes have two bands. Each of those different bands on the gel is assumed to be coded by a different allele. If an enzyme is dimeric, i.e. it is composed of two different polypeptide chains, and is coded by a single locus; a heterozygote individual would form three bands on the gel

as all probable combinations of the two alleles. Each of those bands is assumed to be couples of peptides combined in all probable combinations available.

Typical banding patterns of a monomeric and a dimeric enzyme coded by a single locus are illustrated in Figure 3. In this illustration, sample 1,3,4,6 denote homozygote individuals whereas sample 2, 5 denote heterozygote individuals. A sample of starch gel electrophoresis results obtained in the current study can be seen in Figure 4. When the gel photo in Figure 5 is interpreted according to the key in Figure 3, it can be concluded that PGM in honey bees is a monomeric enzyme coded by a single locus. Individuals 1, 2, 4, 5, 8, and 10 PGM homozygotes and individuals 3, 6, 7, and 9 are heterozygotes. However, it should be noted that 1st banding pattern in Figure 3 cannot be observed in the gel photos. That is, PGM-*100/100* was found not to exist in honey bees.

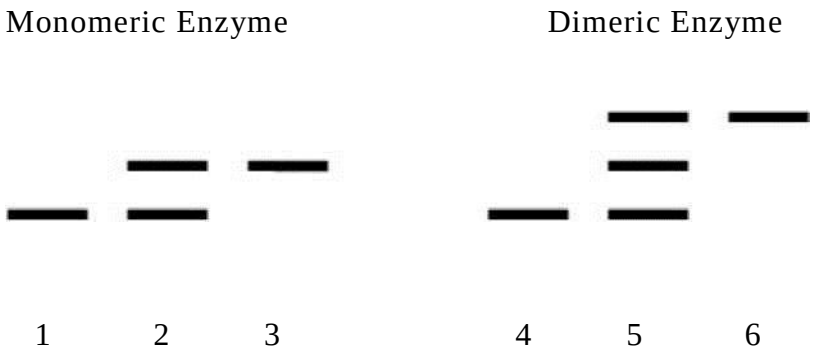

Figure 3. Typical banding patterns of a monomeric and a dimeric enzyme coded by a single locus

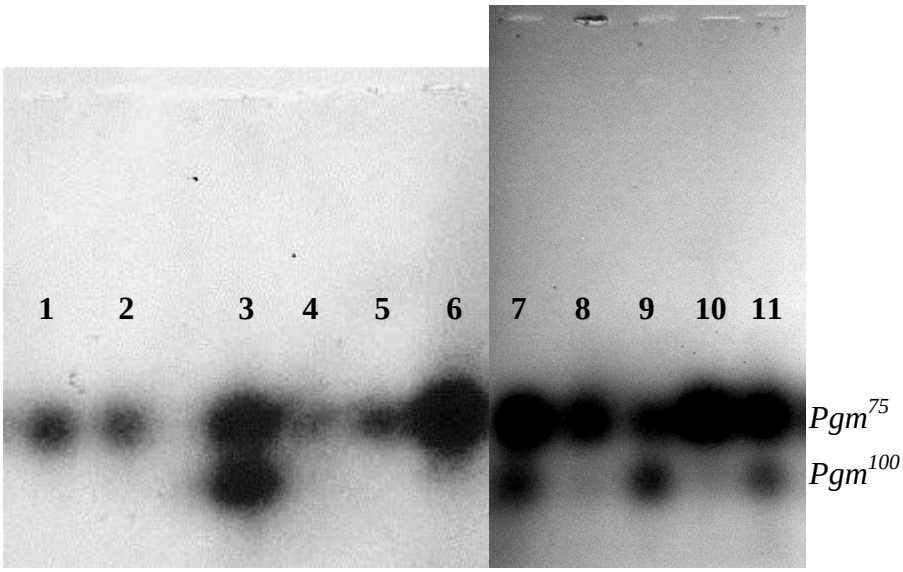

Figure 4. Samples of starch gel electrophoresis results obtained in the current study

2.5. Limitations of protein electrophoresis

Not all amino acid substitutions are detectable by electrophoresis. It is estimated that only about a third of all amino acid substitutions can be detected by the technique (May, 1994, Acquaah, 1992). Such cryptic variation can mask a considerable amount of polymorphisms causing an underestimation of variation. Also it must be noted that only the coding regions of DNA are being interpreted via protein electrophoresis (May, 1994). Electrophoresis protocols available are limited to water-soluble proteins, which leaves out a large group of proteins that are lipid soluble (Zeidler, 2000). Moreover, less than half of all loci are polymorphic (Zeidler, 2000). Polymorphic loci are hard to find specifically in narrow endemic species and others that have experienced

genetic bottlenecks (Parker *et al.*, 1998). The assumption of many statistical analyses used in population genetics is allozymes are different on a minimal level and those differences are selectively neutral, which has been shown to be otherwise for at least some cases (Zeidler, 2000). Finally codominance, another critical assumption of population genetics studies of allozyme frequencies, is not true for all allozymes (Wendel, 1989).

2.6. Statistical Analysis of Allozyme Variation

Basic assumptions of the Hardy-Weinberg Principle were acknowledged for the calculations of genotypic frequencies and expected heterozygosities. Those assumptions are: i) all matings are random; ii) allelic frequencies are conserved between successive generations; ii) no significant migrations occur; and iv) mutation, genetic drift, selection and gene flow are negligible.

Allele frequencies were calculated as the proportion of the observed allele to the total number of alleles in the population using the formula below:

By phenotype frequency, it is meant the proportion of a particular phenotype with respect to the number of all phenotypes in a population. Once the allozyme analysis was done, and heterozygote and homozygote individuals are determined, observed heterozygosity (H_O) was calculated using the formula below:

$$\text{Observed Heterozygosity} = \frac{N_{\text{Heterozygotes}}}{N_{\text{Total}}}$$

Expected heterozygosity (H_E) is an estimation of level of heterozygosity under Hardy-Weinberg equilibrium and is computed based on allelic frequencies gathered from allozyme analysis. POPGENE software on the web (Yeh & Yang, 1999) was used for calculating heterozygosities with Levene's correction which is the same as Nei's (1978) unbiased heterozygosity.

The closeness of the observed genotypic frequencies to those which would be expected under Hardy-Weinberg predictions was detected via goodness-of-fit (*G*) test. The null hypothesis (H_0) is that H_o and H_e do not differ and the population is in Hardy- Weinberg equilibrium. A poor fit is revealed by probabilities less than 1%. Calculation of probabilities for *G* test was also done via POPGENE software (Yeh & Yang, 1999). SPSS 20.0 for Windows was used for the analysis of variance.

CHAPTER 3

RESULTS

3.1. PGM Allozyme Frequency and Overwintering Success

A total number of 356 worker bees from 9 different hives with varying overwintering success were studied in the electrophoretic analysis of PGM. The phenotypes of PGM are designated by using the relative mobilities with the fastest allozyme used as the standard (relative mobility=1.00). The 9 hives were labeled in groups of three as "high success", "intermediate success", and "low success" depending on the ratio of the colony that have survived the winter; more than 80%, 40-60%, and less than 30% survivors, respectively. Allele frequencies at *Pgm* locus for colonies with varying degrees of overwintering success can be seen in Table 3.

Examination of starch gel results reveals that PGM is a monomeric enzyme with one locus in honeybee genome. In previous studies four different alleles had been observed in the electrophoretic analysis of *Pgm* locus; they were named Pgm^{65}, Pgm^{75}, Pgm^{100}, Pgm^{110} according to their relative mobilities with Pgm^{65} having the smallest mobility and Pgm^{110} with the highest. Pgm^{65} and Pgm^{110} had been shown to be the rare alleles having the lowest frequencies as a conjoint result of two successive studies conducted in our laboratory where PGM allozyme frequencies were examined on a monthly basis for a total of 46 months and 3460 worker bees were sampled in total. The results of those two studies confidently points out that alleles Pgm^{75} *and* Pgm^{100} comprise the

99.2% of all *Pgm* alleles observed (Kence *et al.*, 2008).

Table 3. Allele frequencies at *Pgm* locus for colonies with varying degrees of overwintering success

Allele frequencies of *Pgm* as Foraging Flights Begin in Spring 2012		
Overwintering Success	***Pgm*^{75}**	***Pgm*^{100}**
High 1	0.5385	0.4615
High 2	0.5556	0.4444
High 3	0.5408	0.4592
Intermediate 1	0.6176	0.3824
Intermediate 2	0.6667	0.3333
Intermediate 3	0.5526	0.4474
Low 1	0.5732	0.4268
Low 2	0.6000	0.4000
Low 3	0.7250	0.2750

The error bar chart of PGM heterozygosity for colonies with high, intermediate, or low overwintering success was given in Figure 5. The error bar chart shows excess heterozygosity in the colonies with high overwintering success compared to that of intermediate and low overwintering success colonies, which have almost identical means and variation. (Table 4).

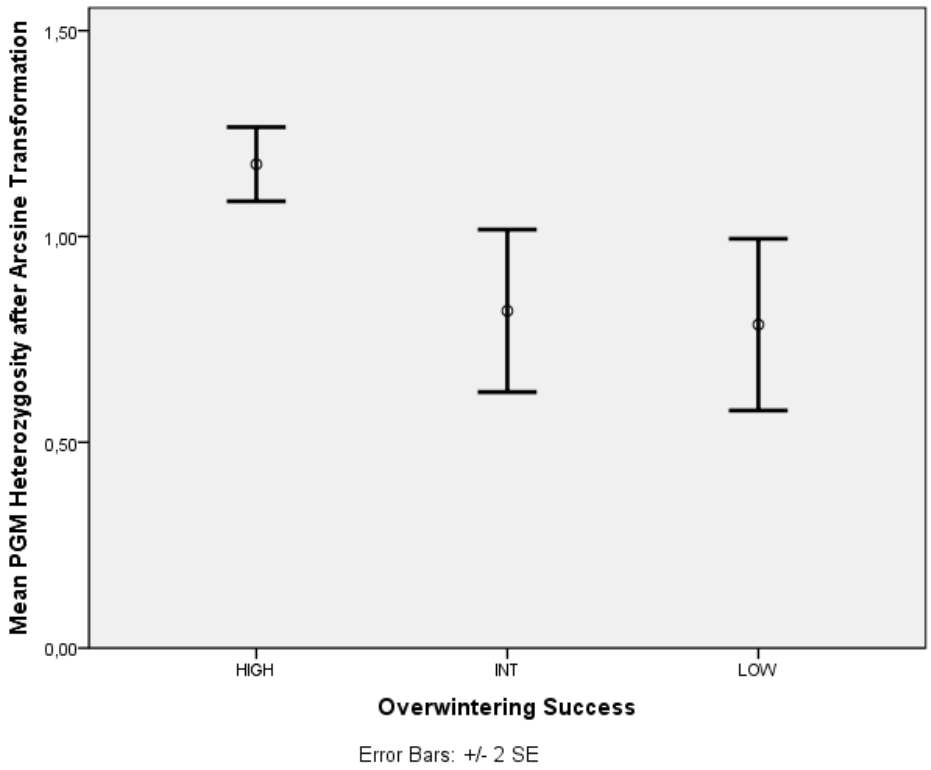

Figure 5. The error bar chart of PGM heterozygosity for colonies with high, intermediate, or low overwintering success

Table 4. Descriptive statistics of PGM heterozygosity data for colonies with varying levels of overwintering success after arcsine transformation

Descriptive Statistics								
PGM Heterozygosity after Arcsine Transformation								
	N	Mean	Std. Dev.	Std. Err.	95% Confidence Interval for Mean		Min.	Max.
					Lower Bound	Upper Bound		
HIGH	3	1.17	.078	.045	.98	1.37	1.10	1.25
INT	3	.82	.171	.099	.39	1.24	.71	1.02
LOW	3	.79	.181	.104	.34	1.23	.58	.93
Total	9	.93	.228	.076	.75	1.10	.58	1.25

Observed and expected frequencies of PGM phenotypes, *G*-test for goodness-of-fit likelihood ratio test (*G*) and chi-square test (χ^2) values for Hardy-Weinberg equilibrium and corresponding probabilities for colonies with varying degrees of overwintering success is given in Table 5. Regardless of the success classes, all of the phenotype frequencies were found to exhibit significant levels of deviations from Hardy-Weinberg equilibrium, which had been reported as a common phenomenon in winter samples of honey bees of different subspecies, namely *Apis mellifera caucasica, A. m. carnica,* and *A. m. meda* (Kence *et al.*, 2008).

Table 5. Observed and expected frequencies of PGM phenotypes, likelihood ratio test (*G*) and chi-square test (χ^2) values for Hardy-Weinberg equilibrium and corresponding probabilities for colonies with varying degrees of overwintering success (d.f.=1).

Observed and Expected Frequencies of Common PGM Phenotypes as Foraging Flights Begin in Spring 2012								
	Observed		**Expected**					
Overwintering Success	***PGM*-75/75**	**PGM-75/100**	**PGM 75/75**	**PGM-75/100**	***G***	***P***	χ^2	***P***
High 1	.077	.923	.923	.923	35.748	.000	27.805	.0001
High 2	.111	.889	.501	.499	36.003	.000	28.016	.0001
High 3	.082	.918	.498	.502	44.259	.000	34.485	.0001
Intermediate 1	.235	.765	.521	.479	16.702	.000	12.456	.0001
Intermediate 2	.333	.667	.551	.449	17.289	.000	12.313	.0001
Intermediate 3	.105	.895	.501	.499	46.898	.000	36.559	.0001
Low 1	.146	.854	.505	.495	28.508	.000	22.017	.0001
Low 2	.200	.800	.508	.492	10.972	.001	8.261	.004
Low 3	.450	.550	.591	.409	3.898	.048	2.574	.109

The heterozygosity values of colonies with varying levels of overwintering success are given in Table 6. There is a significant deviation from expected heterozygosities and Nei's heterozygosities regardless of the overwintering

success indicating that H_o exhibit a differentiation from the ones estimated under Hardy-Weinberg conditions. However, it should be noted that the degree of deviation is greater in colonies with higher overwintering success compared to intermediate and low success colonies. Excess heterozygosity had been shown to be a common property of winter colonies of honey bee and claimed to be a sign of selection (Kence *et al., 2008*); the results of this study shows that having excess PGM heterozygosity can increase fitness in winter colonies.

The Levene Statistic results, which can be found in Table 7, reject the null hypothesis that the group variances are equal.

Results of one-way ANOVA show that PGM heterozygosity levels between groups of colonies with different overwintering success do vary significantly. ANOVA results are listed in Table 8.

Table 6. H_o and H_e of PGM for colonies with varying degrees of overwintering success.

H_o and H_e of PGM as Foraging Flights Begin in Spring 2012			
Overwintering Success	**H_o**	**H_e***	**Nei's****
High 1	.9231	.5035	.4970
High 2	.8889	.4994	.4938
High 3	.9184	.5018	.4967
Intermediate 1	.7647	.4794	.4723
Intermediate 2	.6667	.4488	.4444
Intermediate 3	.8947	.4988	.4945
Low 1	.8537	.4953	.4893
Low 2	.8000	.4923	.4800
Low 3	.5500	.4090	.3987

* H_e was computed using Levene (1949)

** Nei's (1973) H_e

Table 7. Levene Statistic for PGM heterozygosity as Foraging Flights Begin in Spring 2012

Test of Homogeneity of Variances			
PGM Heterozygosity after Arcsine Transformation			
Levene Statistic	d.f. 1	d.f. 2	Sig.
1.907	2	6	.228

Table 8. One-way ANOVA results of PGM heterozygosity levels between groups of colonies with different overwintering success

ANOVA					
PGM Heterozygosity after Arcsine Transformation					
	Sum of Squares	d.f.	Mean Square	*F*	Sig.
Between Groups	.280	2	.140	6.178	.035
Within Groups	.136	6	.023		
Total	.416	8			

3.2. PGM Allozyme Frequency in Manipulated Colonies

360 adult worker bees and 300 larvae were sampled and electrophoretically examined during manipulation experiments. Colonies were manipulated in order to examine the effect of two environmental variables that change with the seasons; temperature and forager originated food availability. The experiments were initiated at the end of the first week of August and ended on September 12th. During this period, a handmade watering system was adjusted on a hive so that the entrance wood receives artificial rain at all times in order to simulate the lack of foragable food in the environment. Another hive was exposed to cold via being placed in a cold room at 4^{o}C in order to mimic winter temperatures. A third hive was left untouched in the apiary as a control. PGM allozyme variation in adult honey bee workers was examined in each colony at the beginning and at the end of the experimental period. Initial and final allozyme frequencies of the adult worker bees that were included in the experiments are listed in Table 9.

At the end of the 4th week of simulated conditions, eggs were marked in all colonies to be followed as larvae; however, eggs that were laid in the "cold" hive never matured into larvae. Thus, in this period, only "rain" versus control larvae were used as samples for allozyme frequency analysis. Later the temperature manipulation experiment was repeated at the end of September. This time the hive was placed in a cold room at 10^{o}C to provide a milder effect on the colony and let them provide better brood care. Eggs were labeled in the colony placed in the cold room and in another colony that was left untouched in the apiary – after now it will be called "hot" hive for convenience. Half of the labeled eggs were exchanged between those hives; so, initially, the plan was to have four distinct conditions: (1) eggs are laid and larvae develop in

the cold hive, (2) eggs are laid and larvae develops in the hot hive, (3) eggs are laid in the cold hive but larvae develops in the hot hive and (4) eggs are laid in the hot hive but larvae develops in the cold hive. However, 4th group did not develop into larvae and the cells were empty when checked for larvae a week later. Numbers of PGM homozygotes and heterozygotes in larvae sampled from manipulated colonies are summarized in Table 13.

Table 9. Allele frequencies at *Pgm* locus for colonies under manipulation.

Allele frequencies of *Pgm* under Manipulation		
	Pgm-75	*Pgm-100*
Rain Initial	.9833	.0167
Rain Final	.5083	.4917
Cold Initial	.9750	.0250
Cold Final	.5333	.4667
Control Initial	.9583	.0417
Control Final	.6667	.3333

Observed and expected frequencies of PGM phenotypes, likelihood ratio test (G) and chi-square test (χ^2) values for Hardy-Weinberg equilibrium and corresponding probabilities for colonies under manipulation were given in Table 10. Results show that PGM phenotypes fit with expected values of Hardy-Weinberg equilibrium when the data from the beginning of the experiments are examined. PGM phenotype frequencies of colonies when sampled at the end of the experiments, on the other hand, deviate from

expected values of Hardy-Weinberg equilibrium significantly. It should be noted that the data coming from initial conditions of the colonies are consistent with the control and the data collected in previous studies in the same time of year. However, samples taken at the end of the manipulation experiments deviate significantly from the control and resemble heterozygosity levels of winter samples even though they were collected on September 12th.

Table 10. Observed and expected phenotype ratios of PGM, likelihood ratio test (*G*) and chi-square test (χ^2) values for Hardy-Weinberg equilibrium and corresponding probabilities for colonies under manipulation (d.f.=1).

Observed and Expected Frequencies of Common PGM Phenotypes under Manipulation								
	Observed		Expected					
	PGM-75/75	PGM 75/100	PGM 75/75	PGM 75/100	*G*	*P*	χ^2	*P*
Rain Initial	.9667	.0333	.9669	.0331	.0169	.8964	.0085	.9263
Rain Final	.0167	.9833	.4959	.5041	73.3876	.0001	55.1634	.0001
Cold Initial	.9500	.0500	.9508	.0492	.0513	.8208	.0261	.8717
Cold Final	.0667	.9333	.4980	.5020	57.9193	.0001	45.0694	.0001
Control Initial	.9167	.0833	.9195	.0805	.1740	.6766	.0900	.7642
Control Final	.3333	.6667	.5518	.4482	20.4289	.0001	14.5633	.0001

The heterozygosity values of colonies before and after manipulation were given in Table 11. There is no significant deviation from H_e and Nei's heterozygosities before the manipulations; however, samples taken after manipulations exhibit a strong deviation from H_e levels. Excess heterozygosity can be explained by PGM heterozygosity having an adaptive value under certain circumstances.

Table 11. H_o and H_e for *Pgm* locus for colonies under manipulation.

H_o and H_e for *Pgm* locus under Manipulation			
	H_o	H_e *	Nei's**
Rain Initial	.0333	.0331	.0328
Rain Final	.9833	.5041	.4999
Cold Initial	.0500	.0492	.0488
Cold Final	.9333	.5020	.4978
Control Initial	.0833	.0805	.0799
Control Final	.6667	.4482	.4444

* H_e was computed using Levene (1949)

** Nei's (1973) H_e

Replicated *G*-test of goodness of fit has three null hypotheses which are; observed heterozygosity does not deviate from expected heterozygosity in individual experiments, the level of deviation is not different between experiments, and heterozygosity of pooled data set does not deviate from the

expected values. The results of replicated *G*-test are summarized in Table 12. Very low *P* values for sum, pooled, and heterogeneity *G*-values indicate that all of the null hypothesis are rejected. That means the PGM heterozygosity deviates significantly from that predicted by Hardy-Weinberg equilibrium in individual experiments, the level of deviation is different between different experiments, and pooled data set also deviates from the expected values of heterozygosity.

Table 12. Replicated *G*-test of goodness of fit results

Replicated G-tests of goodness-of-fit	
Sum of *G*-values	151.9780
d.f.	6
P	10^{-5}
Pooled *G*-value	49.5061
Pooled d.f.	1
P	10^{-5}
Heterogeneity *G*-value	102.4719
Heterogeneity d.f.	5
P	10^{-5}

Table 13. Numbers of PGM homozygotes and heterozygotes in larvae sampled from manipulated colonies.

	PGM75/75	PGM75/100
Rain	7	39
Cold	15	0
Cold to Hot	40	0
Hot	0	60
Control	12	38

CHAPTER 4

DISCUSSION

This is the first study that tests the correlation of PGM polymorphism with the overwintering success and probable environmental cues effecting the seasonal fluctuation of enzyme polymorphism in *Pgm* locus in honey bee (*Apis mellifera*). Seasonal changes in allele frequencies of PGM and phenotype frequencies have been shown in earlier studies in various subspecies of honey bees, *A. m. caucasica, carnica*, and *meda* from different climatic and geographic regions in Turkey (Hadımoğulları *et al*., 2002; Kence *et al., 2008*). Excess PGM heterozygosity observed in winter colonies suggested a selective advantage since observed phenotype frequencies exhibit strong deviation from the heterozygosity levels expected under Hardy-Weinberg assumptions. Correlation of heterozygosity levels in *Pgm* locus of winter honey bee colonies and overwintering success reveals, for the first time, adaptive advantage of excess heterozygosity in *Pgm* locus is at work. Honey bee colonies that survive winter with minimal population loss were shown to have highest heterozygosity levels that are significantly different from heterozygosity levels of colonies that lost half or more of their population size during winter.

Manipulated environmental conditions were provided to test two basic environmental cues related to the change of seasons in nature; temperature and cessation of food influx to the colony via transportation of fresh nectar and pollen by forager bees. The results of the manipulation experiments show a strong effect of cessation of food influx on PGM heterozygosity levels of the

colony. A similar effect was observed when the colony was placed in a cold room at a fixed temperature of 4°C. However, it must be noted that it is virtually impossible to expose a colony to extreme cold and keep the foraging on; thus, all four combinations of two environmental cues could not be tested. Experimentation on larvae via exchanging eggs between cold and hot hives did not result in any larvae. None of the eggs transferred from or into the "cold" hive have developed into larvae when the hive was kept at 4°C. There were no sign of eggs or larvae remains which may be an indication of cannibalism under stress. A similar case reported by Crailsheim *et al.* (2003) that they observed increased cannibalism and decreased brood care under stress involved food shortage (Crailsheim *et al.,* 2003).

4.1. PGM Heterozygosity and Overwintering Success

Analysis of PGM polymorphism in 9 honey bee colonies with different levels of overwintering success has shown that colonies with highest overwintering success have the highest heterozygosity at *Pgm* locus ($P < .05$). The mean PGM heterozygosity of successful colonies was 91% compared to 77.5% in intermediate and 73.5% in low overwintering success colonies.

For a better understanding of the phenomenon at hand, the author wishes to refer to findings of earlier work on PGM polymorphism, its seasonal fluctuation, correlation to division of labor, and difference in the enzymatic activities of homozygous and heterozygous individuals. Even though PGM polymorphism in honey bee has been investigated thoroughly along with many other enzymes for population genetics studies (Nunamaker & Wilson, 1981; Robinson & Page, 1989; Sheppard & Berlocher, 1989), its seasonal fluctuation had remained unnoticed for a long time most probably because sampling honey

bees in winter is not much preferable for its probable hazard to the colonies. Once winter samples of honey bees were obtained it became apparent that PGM heterozygosity levels of colonies in winter were far beyond the Hardy-Weinberg expectations (Hadımoğulları *et al*., 2002); a phenomenon that deserved detailed investigation. Research on the structure of colonies in terms of PGM phenotypes were followed by taking monthly samples for long term monitoring and the results showed a consistent pattern of seasonal fluctuation in *Pgm* locus which could not be observed in other loci studied; PGM heterozygosity is no more than 10% in August and no less than 90% in February (Kence *et al*., 2006). As a result of this curious fluctuation, the question was asked how heterozygosity levels are related to the fitness of colonies. Biochemical analyses revealed significantly higher enzyme activity and glycogen content in heterozygous individuals. (Güldüren *et al*, 2008).

Results of allozyme polymorphism at *Pgm* locus of honey bee colonies with varying levels of overwintering success, when combined with previous findings on seasonal fluctuations in PGM heterozygosity and difference in enzymatic activity of PGM electromorphs, suggest adaptive value of increased PGM heterozygosity in honey bee colonies during winter. The results indicate that honey bee colonies with higher heterozygosity have a higher survival rate through harsh conditions of winter. Consequently, colonies which have larger number of survivors at the end of winter will have advantage over colonies with reduced size at the beginning of spring due to scramble competition for resources such as nectar, pollen, and water.

Here, a follow up question may be "What advantage does increased PGM heterozygosity give to the honey bee colonies for surviving the winter?" Earlier studies on the overwintering behavior and physiology have revealed

some information that can be interpreted together with aforementioned studies in order to come up with a probable answer.

Honey bees have to face with environmental stresses such as limited food and water resources, low temperature, and hypoxia throughout winter. Winter bees do not perform foraging flights as summer bees, however for maintaining stable temperatures in the hive they form clusters (Kronenberg & Heller, 1982) and releasing energy from their flight muscles by carbohydrate breakdown (Stabentheiner *et al*., 2002). Energy demands of thermoregulation and flight were found to be similar as well as metabolic state and glycogen stores of heating bees (bees in cluster) and the foragers (Crailsheim & Panzenböck, 1997). Glycogen reserves of winter bees were shown to be higher than that of summer bees (Crailsheim & Panzenböck, 1997; Güldüren *et al*., 2008). Seasonal variation as well as variable levels of glycogen storage among the layers of winter cluster point out to the contribution of glycogen reserve to the social thermoregulation which is a unique adaptation honey bees have evolved to survive through the winters (Crailsheim & Panzenböck, 1997), which can all be traced back to excess PGM heterozygosity of the colonies in winter.

4.2. Environmental Cues in the Regulation of PGM Heterozygosity

Natural selection may have caused colonies to keep "becoming more heterozygous in winter" as a trait; yet, how do the bee colonies manage to adjust their heterozygosity levels to the seasonal change has remained a question to be answered. This puzzle can be divided into easier-to-solve fractions: "Which elements of seasonal change are the signals for the bees to trigger such alteration in PGM polymorphism?" and "Which molecular and/or behavioral mechanisms are employed for the transformation of a colony with

extremely low heterozygosity into one with extremely high heterozygosity when the individuals of the colony are all siblings?"

In the current study, first fraction of the puzzle was tried to be answered. Samples taken on the first day of the experiment prior to any manipulations showed low PGM heterozygosity consistent with earlier observations of summer colonies; i.e. 3.3% for rain colony, 5.0% for cold colony, and 8.3% for the control. After 36 days of manipulation PGM heterozygosities were recorded as 100% for rain, 93.3% for cold and 66.6% for control colony. It is clear that the PGM heterozygosity increased in all colonies over time. According to the results of *G*-test no significant deviations from the heterozygosity levels expected by Hardy-Weinberg equilibrium were observed in any of the initial samples. Phenotype frequencies of final samples from all colonies deviated significantly from Hardy-Weinberg ratios. The largest deviation was in the rain hive ($G = 73.39$, d.f. = 1, $P < .001$), cold hive was in the middle ($G = 57.92$, d.f. = 1, $P < .001$), and least amount of deviation was observed in the control hive ($G = 20.43$, d.f. = 1, $P < .001$). Here, it should be emphasized that the levels of heterozygosities in manipulated hives were significantly higher than that of the control hive. As a matter of fact, final PGM heterozygosity in the control hive was consistent with the earlier observations of PGM phenotype frequencies made in September months of different years with different subspecies of honey bee (Güldüren, 2007).

Second fraction of the puzzle remains to be answered. Current study alone does not provide an explanation to this question, yet the results here can be integrated with earlier data to infer one.

The significant difference obtained by cutting the food influx in one of the colonies sitting side by side in the apiary caused a significant difference in the heterozygosity levels of the colonies. These results imply that the effect of cessation of food influx on PGM heterozygosity is independent of the temperature which can naturally be observed. The temperature, of course, decreases as the season turns from summer to autumn. However, a much more sharp change can be observed in the amount of food around the apiary since most plants flower between late February and early August depending on climatic regions (Fitter & Fitter, 2002). Foragers can be observed from March to November depending on the weather conditions, however the greatest amount of foraging takes place May to September (Visscher & Seeley, 1982), which is consistent with the availability of flowers. If a colony keeps the same proportion of foragers despite decreasing resources, cost of foraging exceeds the benefit. This information suggests that foraging effort and the forager proportion must be regulated in a pattern to follow food availability in order to obtain maximum gain from foraging. Regulation of foraging is quite complex since foraging must be adjusted depending on the rate of nectar influx and available empty storage in the combs, which build up into a "complex mathematical function" of the foraging success of thousands of other foragers and content of thousands of cells in the hive (Seeley, 1989). Seeley (1989) made a remarkable observation that sheds light on the behavior which can be the principle mechanism that provides a cue for the regulation of foraging via simplifying the complex mathematical function. Seeley (1989) states that foragers need to "unload" their cargo to food storer bees in order to be able to go for another round of foraging flight and the time pass while the forager waits before unloading to food storers cause formation of a queue and the length of this queue is a reliable and sensitive indicator of a colony's nutritional status. Inevitably, queue length is the "visible" result of the mathematical function formed by the ratio of the rate of arrival of foragers at the hive (arrival

rate) and the rate of recipient food storers at the nectar delivery area (service rate). Arrival rate and service rate are functions of the colony's nectar intake rate and its empty comb area, respectively (Seeley, 1989).

Fifteen years later from Seeley's excellent observation on the queue length, another study pointed at a chemical that seemed to be responsible in the regulation of onset of foraging in adult worker bees and resulting in an alteration in the proportion of foragers in the colony (Leoncini *et al*., 2004). The detected chemical was ethyl oleate which was shown to inhibit the behavioral maturation of young bees at least in part when abundant and lead to accelerated behavioral maturation of some younger bees in response to reduction in number or to the absence of foragers (Leoncini *et al*., 2004). Another important finding of the study, which lets us connect it with the observation of Seeley (1989), is the production, localization and the distribution of ethyl oleate. The highest concentration of ethyl oleate was found in crop where its synthesis takes place (Leoncini *et al*., 2004). Crop is a specialized foregut of forager honey bees where the nectar is temporarily stored without digestion and then circulated throughout the colony by trophallaxis. When combined with Seeley's (1989) observation on the forager bees' unloading queue length it can be seen that as the foragers wait longer in the queue, greater amounts of ethyl oleate will be synthesized in the crop resulting in an inhibition of behavioral maturation of younger worker honey bees. It is basically a cue telling the whole colony to stop maturing into foragers since the arrival rate has surpassed the service rate. On the other way around if the foragers do not have to wait for unloading, ethyl oleate production will be reduced and a greater number of young worker bees will mature into foragers as a result of decreased ratio of arrival to service rate.

What have been summarized so far may look remote to the objective of this study; testing the environmental cues related to seasonal change in PGM heterozygosity until a final study is mentioned and combined with the findings of Seeley (1989), Leoncini *et al.* (2004) and the current study. For an investigation of PGM heterozygosities in different castes of honey bee colonies, foragers and nurses were sampled separately. Results showed that even though sampled in summer, there was heterozygosity excess of PGM among foragers; whereas, nurses were predominantly homozygous (Kence *et al., 2008*). All the studies aforementioned can be used to deduct that ethyl oleate may be a signal molecule, the concentration of which is determined by the ratio of loaded foragers to recipient storers, causing the colony to produce less foragers when abundant and more foragers when scarce; and since foragers are mostly heterozygous individuals as opposed to their nurse siblings, here it is speculated that ethyl oleate may be the principle signal molecule that regulates PGM heterozygosity along with many other aspects of division of labor and transformation of the colony from summer bees to winter bees.

Even if a candidate signal molecule is put forward, still there has to be a molecular mechanism that receives the environmental cue via ethyl oleate or other signals and in response alters the expression of *Pgm* locus. The molecular mechanism leading resulting in the observed PGM polymorphism can be either genetic or epigenetic. Genetic mechanisms include; multilocus system I where independent proteins with the same enzymatic activity are coded by different genes, multilocus system II where different subunits of a polymeric enzyme are coded by multiple loci, multilocus-polymeric system where enzymes display a series of polymers that consist of identical subunits, and allozyme system where alleles of a single locus code for the variants of the enzyme (Zeidler, 2000).

A single locus is proposed for *Pgm* and thus the polymorphism most likely results from either an allozyme system or epigenetic mechanism. Alternative splicing of mRNA, RNA editing, protein methylation, differences in the folding pattern of peptides and other epigenetic mechanisms should be investigated to shed light on the molecular mechanism resulting in PGM polymorphism.

CHAPTER 5

CONCLUSION

This is the first study that tests the correlation of PGM heterozygosity with the overwintering success and probable environmental cues effecting the seasonal fluctuation of PGM phenotype frequencies in honey bee (*Apis mellifera*).

Honey bee colonies with highest overwintering success were shown to have the highest heterozygosity at *Pgm* locus ($P < .05$). This result indicates that honey bee colonies with higher heterozygosity have a higher survival rate through harsh conditions of winter. Colonies with larger population size at the end of winter will have a selective advantage over colonies with reduced population size in spring.

Higher glycogen reserves of winter bees (Crailsheim & Panzenböck, 1997) seems to be a result of increased enzymatic activity of PGM heterozygotes. This may be the key feature of winter bees that lets them alter their metabolism to store glycogen more efficiently which will be of crucial energy source for honey bees in winter cluster for the thermoregulation of the hive (Crailsheim & Panzenböck, 1997; Güldüren *et al*., 2008).

Experiments with manipulated environmental conditions revealed a strong effect of cessation of food influx on PGM heterozygosity levels in the honey bee colonies. The combined effect of low temperature and lack of food influx was also found to be similar to cessation of food influx alone; however, it was not possible to test the individual effect of temperature manipulation on PGM

heterozygosity since manipulated or natural harsh cold causes the honey bees to stop foraging.

Increased waiting time in the queue for the foragers to unload nectar to storers had been proposed as a cue (Seeley, 1989). Ethyl oleate produced in the crop of foragers works as a signaling molecule that limits number of foragers in the hive via inhibiting the behavioral maturation of young worker honey bees (Leoncini *et al*., 2004). Here, a greater amount of ethyl oleate is anticipated to be synthesized in the crops of foragers as they wait longer in the queue. Seeley's (1989) behavioral and Leoncini *et al.*'s (2004) biochemical observations may be the components of a complex feedback mechanism that regulates PGM heterozygosity along with many molecular, physiological, and behavioral traits of honey bees as the seasons change.

In further analysis, effects of ethyl oleate on PGM heterozygosity should be tested with experiments that combine molecular data with behavioral observations for better understanding the extent of the phenomenon. In addition to that, molecular mechanisms resulting in PGM polymorphism need to be investigated since the results would provide greater insight on the issue.

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APPENDIX A

Reagents Used in the Electrophoretic Analyses

Name of the Reagent	Brand Name
Starch	Sigma
Agar	Sigma
Glucose-1-Phosphate	Sigma
Glucose-6-Phosphate Dehydrogenase	Sigma
$MgCl_2$	Sigma
NADP	Sigma
PMS	Sigma
MTT	Sigma
Tris	Sigma
EDTA	Sigma