CAUSES AND CONSEQUENCES OF SEASONAL VARIATION OF PHOSPHOGLUCOMUTASE (PGM) ENZYME POLYMORPHISM IN HONEYBEES (*Apis mellifera L.*) OF TURKEY

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ABSTRACT

CAUSES AND CONSEQUENCES OF SEASONAL VARIATION OF PHOSPHOGLUCOMUTASE (PGM) ENZYME POLYMORPHISM IN HONEYBEES, (*Apis mellifera* L.) OF TURKEY

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Phosphoglucomutase (PGM) is one of the central enzymes in energy metabolism at a branch point at the head of the metabolic pathway leading into glycogen metabolism, the pentose shunt, and the main glycolytic cycle, catalyzing the reversible interconversion of glucose-1-phosphate to glucose-6-phosphate.

Whole year, month to month analysis of pattern of allozyme variation at *Pgm* and *Hk* *l*oci in *Apis mellifera* L. from three provinces; Kırklareli, Artvin, and Hatay revealed that there is significant seasonal variation of allozyme frequencies at *Pgm* locus ($P<0.001$). The difference in genotype frequencies between summer and winter samples is apparent in *Pgm*, whereas at *Hk* locus, which is analyzed as a control there

is seasonal variation in genotype frequencies. Biochemical measurements of the enzyme activities and glycogen content of different *Pgm* genotypes were performed to determine the effect of different *Pgm* genotypes on the physiological performance of the honeybees and it was observed that both enzyme activity and glycogen amount is higher in heterozygote individuals which are in high frequency during winter months ($P<0.0001$). Furthermore, PGM enzyme activity and glycogen content was found to be significantly correlated. These findings clearly demonstrate that biochemical differences between different *Pgm* genotypes have functional correlates that lead to significant variations in glycogen content of the honeybees and may have adaptive consequences.

Keywords: Phosphoglucomutase (PGM), Allozymes, Seasonal Variation, Enzyme activity, *Apis mellifera*.

ÖZ

TÜRKİYE BAL ARILARINDA (*Apis mellifera* L.) FOSFOGLUKOMUTAZ (PGM) ENZİM POLİMORFİZMİNİN MEVSİMSEL DEĞİŞİMİNİN NEDENLERİNİN VE SONUÇLARININ ARAŞTIRILMASI

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Fosfoglukomutaz enzimi (PGM), glikojen metabolizması, pentoz fosfat yolu ve ana glikolitik koridor arasındaki kavşak noktasında bulunan, enerji metabolismasındaki önemli enzimlerden biri olup sözü geçen metabolik yolda, glukoz-1-fosfat ve glukoz-6-fosfat arasındaki dönüşümü katalizlemektedir.

Hatay, Artvin ve Kırklareli illerinden yıl boyunca her ay alınan toplam 2540 örnekle yapılan allozim çalışmaları sonunda *Pgm* genotip frekanslarının yaz ve kış populasyonlarında mevsimsel varyasyon gösterdiği ve Hardy-Weinberg dengesinden anlamlı ölçüde saptığı ($P<0.001$), kontrol olarak çalışılan *Hk* lokusunda ise alel frekanslarında mevsime bağlı bir değişim olmadığı belirlenmiştir. Farklı *Pgm* genotiplerinin balarılarının fizyolojik performansına etkisini belirlemek amacıyla bu

genotiplerin (*Pgm-75/75* ve *Pgm-75/100*), PGM enzim aktivitesi ve glikojen miktarları belirlenmiş ve kış aylarında frekansı yüksek olan heterozigot bireylerde PGM enzim aktivitesinin ve glikojen miktarının, homozigot bireylere göre önemli ölçüde yüksek olduğu gözlemlenmiştir ($P<0.0001$). Elde edilen bu veriler balarılarında farklı *Pgm* genotiplerinin biyokimyasal ve fonksiyonel olarak birbirinden farklı olduğunu ve bu farklılıkların balarılarının glikojen miktarını önemli ölçüde etkilediğini göstermektedir.

Keywords: Fosfoglukomutaz**,** Allozim, mevsimsel değişim, enzim aktivitesi, *Apis mellifera*.

To

All who are willing to perceive and comprehend the nature of probabilities in nature…

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CHAPTER 1

INTRODUCTION

1.1. Enzyme polymorphisms and adaptation

One of the central issues in evolutionary genetics concerns and questions whether the genetic variation in natural populations lead to significant biochemical, physiological and functional differences that affect the fitness of the individuals in a population (Watt, 1994; Mitton, 1998; Eanes, 1999; Dahlhoff and Rank, 2000; Verrelli and Eanes 2000, 2001a; McMillan *et al*., 2005). The arguments dealing with the adaptive and evolutionary significance of enzyme polymorphisms date back to several decades and have led to the "Neutralist-Selectionist controversy" after the introduction of the "neutral theory of molecular evolution" to population biology literature by Motoo Kimura in late 1960s (Kimura, 1968; Clarke, 1970). Kimura's neutral theory of molecular evolution is based on the hypothesis that majority of the genetic variations (both at the DNA and protein level) in natural populations do not affect the fitness of individual organisms and are "selectively neutral" (Kimura, 1983). Furthermore, the theory also predicts that the amount of genetic variation in natural populations would not be high because high levels of variation brings a cost namely "genetic load" which in high amounts may lead to the extinction of the population (Johnson, 1973; Kimura, 1983).

Since the development of electrophoretic methods large amount of data have accumulated revealing high levels of genetic variation in natural populations. In particular, electrophoretic studies have revealed the existence of a high degree of genetic variation, (about 30 %) at enzyme loci (Lewontin, 1974; Johnson, 1977). However this high percent of genetic variation is still an underestimate because electrophoretic techniques can not detect and resolve the total amount of genetic variation at enzyme loci (Bernstein *et al*., 1973; Scozzarri *et al*., 1979). It has been calculated that among all possible amino acid substitutions in a protein only one third can lead to differences that can alter electrophoretic behavior (Lewontin, 1974). For instance, application of heat denaturation techniques to the electrophoretic analysis of allozymes have revealed the existence of even more alleles (electrophoretically undetected or "cryptic") that differ in their heat stability at the xanthine dehydrogenase and octanol dehydrogenase loci in *Drosophila virilis* (Bernstein *et al*., 1973) and temperature sensitivity at phosphoglucomutase (*Pgm*) locus in *Culex pipiens* (Scozzarri *et al*., 1979). Therefore, the majority of the genetic variation at enzyme loci is still invisible to the electrophoretic analyzes. Actually the level of polymorphism at enzyme loci is much more than predicted by the neutral theory of evolution and enough to question the concept of "genetic load" (Johnson, 1977).

Another theory that implies selective neutrality of the enzyme polymorphisms is the *theory of metabolic flux* which has suggested that differences in the biochemical properties of a single enzyme would have no or undetectable effects on the outcome of the pathway because metabolic pathways are controlled by many structural loci that interact with each other in complex regulatory mechanisms (Kacser and Burns, 1981). Therefore, according to this hypothesis the effect of only one enzyme on the physiological functions or adaptive ability of an organism can not be significant. In other words, electrophoretically different enzyme variants have no selective significance, meaning that they only constitute "molecular noise" (Eanes, 1999). Furthermore, the theory of metabolic flux also hypnotizes that significant differences in the biochemical and kinetic properties of an enzyme are required for that enzyme to lead to detectable changes in the metabolic pathway (Kacser and Burns, 1981).

The theory of metabolic flux is partly based on the degeneracy of the genetic code (Johnson, 1973). However, although the codon degeneracy implies that enzyme polymorphisms are most likely to be undetected by natural selection, it does not state that they have no adaptive consequences (Eanes, 1999)

The theory also emphasizes the significance of the *in vitro* and *in vivo* condition differences implying that the *in vitro* detected biochemical differences among enzyme variants in terms of specific activity (V_{max}), Michaelis constants (K_m) and thermostability may not be as those "employed" by the organism, and consequently can not alter the metabolic functions (Kacser and Burns, 1981; Middleton and Kacser, 1983). This is an important argument against the selective nature of biochemical differences in allozyme variants since it considers that the composition of the cellular environments in which the enzymes function in organisms can not be formed by experimental conditions in which the measurements are performed. However, *in vivo* activity measurements of 6-phosphogluconate dehydrogenase (*6-Pgd*) and glucose-6-phosphate dehydrogenase (*G-6-pd*) allozymes after differential radio-labeling have revealed that the different genotypes of *6-Pgd* and *G-6-pd* show variations in *in vivo* carbon flux rates through the pentose shunt and these variations are in agreement with the results obtained from *in vitro* activity measurements of both enzymes (Cavener and Clegg, 1981). In other words, *in vivo* measurements of enzyme activity have been found to be consistent with the *in vitro* activity assays in a predictable pattern. In addition, it has been documented that different genotypes of *6-Pgd* are significantly associated with the relative amount of lipid and protein synthesis in *D. melanogaster* and these variations at *6-Pgd* enzyme locus have led to fitness differences providing an answer to the argument of theory of metabolic flux by demonstrating *in vitro* kinetic properties of allozymes may be consistent with the *in vivo* differences in metabolic functions (Cavener and Clegg, 1981).

For years, allelic enzyme variants (allozymes) have been used extensively in population genetic studies especially to analyze evolutionary mechanisms and phylogenetic relationships with the assumption that the observed variation is neutral. On one hand this assumption appears to be true in some instances (Kimura, 1983), on the other hand, several studies have shown that there are significant physiological and fitness consequences of the genetic variation at enzyme loci and some allozyme variability may have functional correlates. For example phosphoglucose isomerase (*Pgi*) polymorphisms in willow beetle, *Chrysomela aeneicollis* (Dahlhoff and Rank, 2000) and *Colias* butterflies (Watt *et al*., 1983, 1985), amino acid polymorphisms at phosphoglucomutase (*Pgm*) locus in *Drosophila melanogaster* (Verrelli and Eanes, 2001a), glucose-6-phosphate isomerase (*Gpi*) allelic variants in sea anemone *Metridium senile* (Hoffman, 1985). Koehn *et al*., (1980) have revealed that two allozyme variants at the leucine amino peptidase (*Lap*) locus in common mussel, *Mytilus edulis* show significant variation in their relative catalytic efficiencies, K_{cat}. The variation in biochemical properties, in turn, differentially affect the construction of cellular free-amino acids during periods of adaptation to hyperosmotic stress, and consequently affect the relative viability of organisms carrying different alleles (Moore *et al*., 1980). Another example is the α-glycerophosphate dehydrogenase (α-*Gpdh*) locus in *Colias* butterflies which is highly polymorphic for allozyme variants. The kinetic efficiencies (K_m) of the allozymes are differentially affected by temperature and these variations in enzymatic activity show correlations with the allelic frequency distribution in natural habitats differing in temperature characteristics impliying that allozyme frequencies can modulate adaptive responses to climatic changes associated with geographical range (Johnson, 1976). Dahlhoff and Rank (2000) focused on the effect of environmental temperature changes on the functional properties of phosphoglucose isomerase (*Pgi*) genotypes and the expression of 70-kDa-class heat shock proteins and showed that the geographic distribution of the different *Pgi* genotypes is correlated with thermal adaptation to local temperatures and there are functional (in biochemical properties of different enzyme genotypes) and physiological differences (heat shock protein expression) among the enzyme variants.

In *Colias* butterflies biochemical properties of different *Pgi* genotypes are associated with differences in survivorship, flight behavior, male mating success and female fecundity (Watt 1983, 1994; Watt *et al*., 1985) which are important fitness related traits. Furthermore, it has been observed that in the leaf beetle, *Chrysomela aeneicollis* differences in the functional properties of the *Pgi* allozymes affects the survival of the larvae and thermal behavior of the adult beetles (McMillan *et al*., 2005). Hoffman (1981) reported that kinetic differences among allelic variants of *Gpi* in sea anemone, *Metridium senile* differentially modulate glucose metabolism at the glycolysis-pentose-shunt branch point and concluded that *Gpi* variation could contribute to Darwinian fitness in environments where temperature fluctuations occur. In an extensive survey of allozymes involving 30 enzyme systems, Harris (1966) reported that significant functional variations exist in more than 60% of the naturally occurring allozyme polymorphisms in humans and these functional differences are due to the biochemical differences between allozyme variants including kinetic and thermostability parameters, inhibition constants, optimum temperature and pH values.

These studies have shown that protein polymorphisms can have adaptive value and biochemical differences in the kinetic parameters of allelic variants of a single enzyme locus have the potential to influence physiological performance and contribute to the variation in fitness related traits of organisms. Therefore, as stated by Johnson (1973), the combination of data obtained from the studies that analyze the extend of genetic variation with the data on characterization of biochemical, functional, and physiological correlates of enzyme polymorphisms have revealed that at least some of the genetic variation at enzyme loci is maintained by selection. Nevertheless, it is a difficult task to determine the nature of genetic variation and relate this variation to the physiological and functional consequences since there is still a lack of knowledge about the mechanisms of genetic and biochemical pathways leading to the observed highly complex phenotypic variation within and between populations (Johnson, 1973).

This difficultly is further increased by the fact that majority of the genetic variation in populations do not lead to easily detectable differences in function or physiology, as emphasized by Dykhuizen *et al*., (1986) it is hard to describe an example in which variations at an enzyme locus affects metabolic pathways directly by producing "causal" changes in physiology and fitness related traits.

An enzyme is only a single member of a highly complex metabolic network of connected pathways in which multiple enzymes function and affect each other by regulatory components (Dykhuizen *et al*., 1986). The effect of an individual enzyme variant on such a complex system has been generally assumed to have no significance (Kacser and Burns, 1981). The basis of this argument is that selection must act on the reproductive fitness of organisms (Middleton and Kacser, 1983). What affects the fitness of the organism is the physiological output of the genetic variation at the overall metabolic reactions rather than the individual enzymatic steps within the metabolic pathway (Middleton and Kacser, 1983). However, it has been argued that all enzymes in an organism may not be affected by the selection at equal degrees and the enzymes that have direct or indirect regulatory roles in energy flow through metabolic pathways are particularly more "sensitive to selection pressures" (Johnson, 1973). Watt (1985) has stated that loci that play central roles in energy metabolism may experience greater intensity of selection than loci secondary to energy processing. In the allozyme survey of coot clam, *Mulinia lateralis*, it has been observed that among the 15 enzyme loci studied, only the variations in enzymes that function in glycolysis and protein catabolism lead to significant differences in growth rate (Koehn *et al*., 1988). Determination of the catalytic properties of allozyme variants by biochemical characterization and the incorporation of the amino acid sequence data to the electrophoretic and biochemical analyzes have lead to the understanding that enzyme polymorphisms at branching pathways can be the "targets" of selection because they can "potentially" cause changes in the flux (Eanes, 1999 ;Verrelli and Eanes, 2001a).

In their review article "Evolutionary Physiology" Garland and Carter (1994) emphasized the necessity that genotypic and biochemical variation at enzyme loci should reflect themselves at the level of whole organism so that selection can act on them. In other words, allozymes that differ in functional properties should also lead to differences in the rate of flux or the efficiency of flux, so that the genetic variation at enzyme loci can affect metabolic pathway characteristics which in turn directly or indirectly influence fitness by affecting energy supply, production, and use. The increase in the efficiency and the rate of the flux may change the patterns by which metabolic energy is allocated on different physiological activities and may allow the organism to invest more energy to fitness related traits such as viability and reproductive success (Garland and Carter, 1994).

Integration of studies in population genetics with the physiological and biochemical studies have led to the identification and comprehension of correlations between genotypic or allelic frequencies and environmental or ecological factors, such as habitat temperature, seasonality, latitude, or altitude (Eanes, 1999; Verrelli and Eanes, 2000, 2001a). Organisms respond to the changes in environmental factors in a variety of ways including physiological, behavioral, and morphological adjustments to counter its effects and maintain normal functioning (Watt 1983, 1994; Watt *et al.* 1985; Dahlhoff and Rank, 2000; McMillan *et al*., 2005). The ability of individuals to respond and adapt to these changes is due to the genetic variation that provides physiological "plasticity" in a changing environment (Wu, 1998). By providing metabolic flexibility to the fluctuations in environmental conditions enzyme polymorphisms may increase the adaptability of the organisms (Johnson, 1973).

1.2. Phosphoglucomutase and Its Role in Energy Metabolism

Phosphoglucomutase (PGM; EC 2.7.5.1) is one of the central enzymes in energy metabolism, which resides at a branch point of the glycolytic pathway leading into the glycogen synthesis, the pentose shunt, and the main glycolytic corridor and

catalyzes the reversible interconversion of glucose-1-phosphate and glucose-6-phosphate. (Ray and Roscelli, 1964; Ray and Peck, 1972; Verrelli and Eanes, 2000). PGM plays a key role in directing and regulating the metabolic flux of energy metabolism in all cells (Ray and Peck, 1972). Schematic representation of the basic reactions at the branch point of energy metabolism is given in Figure 1.1.

PGM functions both in glycolysis and gluconeogenesis and generates phosphorylated compounds for the subsequent catabolic reactions that produces metabolic energy or biosynthetic reactions that leads to the synthesis of carbohydrates. (Ray and Roscelli, 1964).

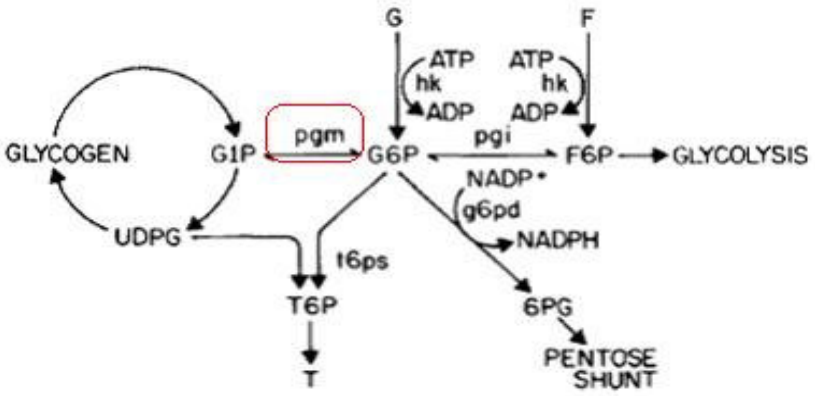

Figure 1.1. Schematic representation of the basic reactions at the branch point of the glycolytic pathway leading into the glycogen synthesis, the pentose shunt, and glycolytic corridor (from Carter and Watt, 1988) Abbreviations: ADP, ATP; adenosine di- and triphosphate, F; fructose, G; glucose, 1-P 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.

PGM is a very widely used enzyme in electrophoretic studies for the analysis of genetic variation in natural populations and it has been determined that *Pgm* locus show considerable polymorphism in almost all species (Nevo and Beiles, 1988; Cicchetti *et al*., 1990) including honeybees (Sylvester, 1986; Sheppard and Berloher, 1985; Kandemir *et al*., 2000). In studies carried out with *Drosophila melanogaster* it has been demonstrated that most of the variation in amino acid polymorphisms at *Pgm* locus determined by geographical variations as adaptive responses to the different climatic conditions (Verrelli and Eanes, 2000). The analysis performed to identify the association of the activity of different *Pgm* genotypes with the thermostability and glycogen concentrations has revealed that there is a positive correlation with PGM activity and glycogen content in *D. melanogaster* (Verrelli and Eanes, 2001b). Studies of allozyme variation at *Pgm* locus in Pacific oyster, *Crassostrea gigas* has also been shown to be one of five loci that contribute to the positive correlation between multiple-locus heterozygosity and adult body weight (Fujio, 1982). Allozyme analysis of a polycheata worm, *Alvinella pompejena* have revealed that *Pgm* locus is highly polymorphic compared to the other enzyme loci that function in glycolysis and Krebs cycle (Jollivet *et al*., 1995). Thermostability and enzyme kinetic analysis of different homozygous genotypes of *Pgm* have demonstrated that there is significant difference between alleles in terms of their thermostability which in turn affect the distribution and population structure of the *Alvinella pompejena* (Piccino *et al*., 2004). The plastid PGM in plants is essential for the synthesis and degradation of starch and other storage products in seeds and deficiencies in PGM activity causes resulted in the production of a "starchless" phenotype in Arabidopsis (Casey *et al*., 1998). In field mouse (*Apodemus sylvaticus*) change in genotype frequencies at *Pgm* locus under hunger stress or food shortage and a correlation between the survival of individuals and different *Pgm* genotypes have been demonstrated (Leigh, 1977). PGM deficiencies lead to glycogen storage diseases in humans (Sugie *et al*., 1988).

These studies converge on the conclusion that PGM is one of the important regulatory enzymes of the carbohydrate metabolism of organisms of many taxa and amino acid polymorphisms at this locus may have physiological consequences that

can alter the physiological performance and adaptability of the organisms. In support of this conclusion is the high amount of variation observed at *Pgm* locus of various species. When evaluated from evolutionary point of view, the general expectation is that the loci coding for enzymes participating to the most vital cellular functions are expected to have low rates of amino acid substitutions (Nei and Kumar, 2000). Therefore, the high amount of allozyme polymorphism at *Pgm* locus may be due to its adaptive consequences and maintained by selection.

1.3. Allozyme Polymorphism and its Relation with Flight Metabolism and Capacity in Insects

It has been documented by Candy *et al*., (1997) that the flight of insects is accomplished with high metabolic costs because it requires the increased activity of several tissues and the most rapidly contracting muscles are insect flight muscles. Endothermic insects, such as honeybees, can reach metabolic rates that are 100 times higher than the resting state (Kammer and Heinrich, 1978; Fewell and Harrison, 2002). Furthermore, it has been reported that >90% of O_2 consumption in the organism is allocated to the contraction of the flight muscles during flight (Crabtree and Newsholme, 1975; Suarez *et al*., 1996). Also, the elevation of the thoracic temperatures and activity during flight can cause further increase in metabolic rates up to 50-100 folds (Kammer and Heinrich, 1978; Casey and Ellington, 1988). This high metabolic rate during flight is provided with the increased activity of the certain glycolytic enzymes functioning close to their "maximal flux capacity" (Suarez, 2000).

In the case of honeybees, in addition to the high energy requirement of the flight activities, forager bees return to the hive with nectar or pollen loads after their flight. These loads can approach to 80% of body weight of the honeybee (Winston, 1987; Fewell and Harrison, 2002). Also, a specialized group of worker bees named as undertakers carry dead bees away from the hive meaning that, they carry loads equal to their body mass during flight (Coelho, 1991).

Carrying such a load during flight increases the metabolic cost by 40% for honeybees (Fewell and Harrison, 2002). Honeybees maintain such a high metabolic output by allocating high amounts of energy to flight (Wolf *et al*., 1989). This is, in turn significantly correlated with another source of variability of flight metabolism; the polymorphism at enzyme loci that participate in the regulation of energy flow. For example as a result of the analysis of electrophoretic variations at three flight related enzyme loci δ-glycerophosphate dehydrogenase (*δ-Gpdh*), glucose-6-phosphate dehydrogenase (*G6pd*) and phosphoglucomutase (*Pgm*) of light-brown apple moth, *Epiphyas postvittana* moths, only PGM was found to be polymorphic among the enzymes studied and this high polymorphism at *Pgm* locus was shown to be correlated with the variation in the flight duration of the moths (Gu, 1991). This result is further supported with the artificial selection studies which demonstrated that selection on flight capacity resulted in significant genotypic differentiation (Gu, 1991). Other studies correlated the variations in insect flight metabolism with polymorphism in several enzyme loci involved in cellular respiration and energy metabolism such as glycerol-3-phosphate dehydrogenase in *Drosophila* (Barnes and Laurie-Ahlberg, 1986), phosphoglucose isomerase in *Colias* butterflies (Watt, 1983).

The source of energy and the metabolite utilized as the fuel for flight may show variations depending on the species (Beenakkers *et al*., 1984; Candy, 1989). Some insects metabolize lipids, others use carbohydrates or proteins as fuel for flight and some use both. For example, sugar is the main source of energy for male bumble bees (*Bombus terrestris* L.), and the amounts of lipids and glycogen are negligible (Surholt *et al*., 1991). Analysis of flight ability and duration in two species of flies, *Drosophila funebris* and *Lucilia sericata* have revealed that glycogen reserves are of primary importance in the physiology of flight in these flies and can become a limiting factor during continuous flight determining the duration and rate of the flight (Williams *et al*., 1977). The importance of carbohydrate degradation and effect of variation in activity of enzymes that regulate carbohydrate metabolism is further

supported by additional studies. It has been reported that the degradation of the carbohydrates is an important step that lead to the raise of the thoracic temperature of insects for the initiation of flight (Beenakkers, 1969). And for many insect species the duration of flight has been found to be significantly correlated with the amount glycogen available (Miquel, 1971; Neukirch, 1982).

Similarly, for honeybees the oxidative fuel for flight is carbohydrates mainly glycogen (Sacktor, 1970; Candy, 1989). Honeybees obtain carbohydrates directly from the collected nectar or honey (Candy, 1989). Comparison of the physiology of the drones and workers have clearly demonstrated the significance of glycogen reserves and the higher activity of the metabolic enzymes required for extensive flight activities of the worker bees as they age for foraging (Hrassnigg and Crailsheim, 2005). It was determined that the total glycogen stores in a forager bee would enable a flight distance of 335 m, and for drones foraging distance reduces to 230 m (Panzenböck and Crailsheim, 1997). This is an important finding showing the difference in efficiency of flight metabolism between drones and forager bees. The glycogen content of honeybees increase during development as they age from larvae to foraging bee (Neukirch, 1982; Kunert and Crailsheim, 1988). Foragers have the highest amount of accumulated glycogen in the flight muscles compared to other ages and behavioral stages (Neukirch, 1982). These results are in accordance with the age related division of labor in the hive and the high energy demand of the foragers. Prior to the foraging period, metabolic rates of flight, thoracic enzyme concentrations and the thoracic glycogen levels remain constant during the 1-3 week period when bees work within the hive (Roberts and Eleconich, 2005). With the onset of foraging, flight metabolic rate increases nearly by 15% and this increase is accompanied by a two fold increase in the concentrations of the thoracic glycogen (Fewell and Harrison, 2002). Therefore, variation in physiology and metabolism of flight is correlated significantly with the age dependent behavioral development and the division of labor in honey bee colonies (Neukirch, 1982). In this sense, enzymes participating the carbohydrate metabolism may have particularly more effective roles in the honeybees, because honeybees rely primarily on the carbohydrates for energy

supply, and nutrition plays important roles in caste determination and socially regulated behavioral plasticity and age polyethism of honey bees (Robinson, 2002; Kunieda *et al*., 2006).

1.4. Heterozygosity - Fitness Correlations

Population biologists reported vast amount of data on the higher fitness of heterozygote individuals in studies of protein polymorphism in populations of many species. In majority of these studies a positive correlation is observed between heterozygosity at allozyme loci and some fitness related traits such as survival, egg production, developmental time, and metabolic rate (Watt, 1977; Ginzburg, 1979; Watt *et al*., 1985; Smouse, 1986; Allendorf *et al*., 1986; Gafney, 1990; Mitton, 1993; Zouros, 1993; Pogson and Zouros 1994; David, 1998). Analysis of multilocus allozyme heterozygosity has shown significant positive correlations with the components of fitness in the rainbow trout, *Oncorhynchus mykiss* (Leary *et al*., 1983). Even a single locus can have major impact on the whole animal physiology. Goulson (1993) observed that the genotype at *Pgm* locus affected the length of time for which individual butterflies, *Maniola jurtina* could fly continuously. The author found that in temperatures of 29^{o}C the individual butterflies homozygous for *Pgm-100* can continuously fly for longer intervals than individuals of other genotypes and he concluded that during cold conditions heterozygotes may fly longer than individuals homozygous for *Pgm-100.* However there are also cases in which no significant relationship is observed between fitness components and heterozygosity at allozyme loci (Mukai *et al*., 1974), and other studies reported the presence of a negative correlation (Gains *et al*., 1978).

Understanding the association between heterozygosity and fitness related traits is particularly important for the identification of the mechanisms that maintain high amount of genetic variation in natural populations. Three main concepts that are thought to be related with the increased fitness of the heterozygote individuals have been summarized as: i) effect of inbreeding in populations, ii) heterozygote

advantage at allozyme loci, iii) linkage disequilibrium and "associative overdominance" (Ohta, 1971).

1.4.1. The Effect of Inbreeding

An important hypothesis in literature that is proposed to explain the allozyme heterozygosity-fitness associations is based on the effect of inbreeding on natural populations (Ledig *et al*., 1983). Inbreeding tends to decrease the level of genetic variation and increase the level of genomic homozygosity in natural populations (Hansson and Westerberg, 2002). Reduced genomic heterozygosity together with the expression of recessive deleterious alleles demonstrates itself with the low fitness of the inbred individuals (Charlesworth and Charlesworth, 1987). Several studies have documented the reduction in fitness related traits such as survival rate and reproductive output by using breeding experiments together with pedigree analysis and reported that the reduction in fitness is significantly associated with the inbreeding coefficients (Falconer and Mackay, 1996; Lynch and Walsh, 1998). When inbred individuals with reduced heterozygosity and fitness are grouped in a sample with outbred individuals with high genomic heterozygosity and fitness, a positive correlation between heterozygosity and fitness is observed (Thelen and Allendorf, 2001).

1.4.2. Heterozygous Advantage at the Allozyme Loci

An alternative mechanism that is suggested to explain the heterozygous advantage at allozyme loci considers the increased biochemical efficiency of heterozygote individuals compared to homozygotes and hypothesizes that heterozygotes may produce enzymes with more efficient catalytic properties whereas homozygotes can produce only one form of the enzyme (Mitton, 1997). This hypothesis depends on the idea that enzyme loci, through their effect on the metabolic pathways can

influence the fitness related traits since allozyme heterozygosity decreases the metabolic cost and favors the allocation of more energy to growth, reproduction, viability, developmental processes and consequently lead to increased fitness of individuals with enzyme heterozygosity at multiple loci (Mitton and Koehn, 1975; Koehn *et al*., 1988; Pogson, 1991; Mitton, 1993, 1998). Burton and Feldman (1983) have demonstrated that the catalytic properties of allozymes have direct influence on the efficiency of the energy flow through metabolic pathways. Therefore organisms using the metabolic energy in a more efficient manner are expected to have higher fitness as in the case of the Watt's study of *Colias* butterflies where individuals that are heterozygote for particular alleles of the enzyme phosphoglucose isomerase have increased flight duration and performance leading to increased mating success of the males and increased female fecundity (Watt *et al*., 1985; Watt, 1994).

Several studies have been performed to test the heterozygote advantage at *Mdh* (malate dehydrogenase) locus which catalyses the conversion of oxaloacetate and malate, hence it is an important enzymatic step in the energy metabolism of many organisms (Musrati *et al*., 1998; Borrell *et al*., 2004). Analyses of allozyme variation at *Mdh* locus have revealed that heterozygote individuals show a higher relative fitness than homozygotes (Oostermeijer *et al*., 1995; McGoldrick and Hedgecock, 1997). In honeybees, comparisons of fluctuating asymmetry of wing vein lengths in heterozygote and homozygote individuals has showed that *Mdh* heterozygotes had less fluctuating asymmetry than the homozygotes (Messier and Mitton, 1996). Fluctuating asymmetry is commonly used as a measure of developmental stability and it is calculated by measuring the difference between morphological characters on both sides of bilaterally symmetrical organisms (Van Valen, 1962). Developmental stability or developmental homeostasis is defined as the ability of an organism to "buffer its development against environmental perturbations" to produce the "targeted phenotype" (Waddington, 1942; Vollestad *et al*., 1999). The theoretical assumption underlying in the analysis of fluctuating asymmetry as an estimate of developmental stability is that during development right and left sides of a bilaterally symmetrical organism is affected by the same genetic mechanisms, therefore the

observed phenotypic variability in the morphological characters is caused by environmental heterogeneity or stress factors (Van Valen, 1962; Clarke, 1998; Dongen, 2006). Several studies have reported the presence of a positive relationship between heterozygosity at allozyme loci and different parameters of developmental stability (Mitton and Koehn, 1975; King 1985; Messier and Mitton, 1996; Mitton 1997). Accordingly, it has been suggested that high levels of heterozygosity at allozyme loci increases the ability of the organism to cope with the environmental changes or rapid fluctuations in ecological factors and consequently lead to increase in developmental stability and fitness related traits (Mitton and Koehn, 1975; Mitton 1993)

1.4.3. Linkage Disequilibrium and Associative Overdominance

Another important hypothesis that is developed to explain the increased fitness of heterozygote individuals is the "associative overdominance" hypothesis that states allozymes are selectively neutral markers and the observed heterozygosity-fitness associations result from the linkage disequilibrium between allozyme loci with other deleterious loci (Ohta, 1971; Crow and Simmons, 1983). In other words, heterozygotes "mask" the phenotypic effect of the linked deleterious recessive alleles and this in turn lead to the observation of heterozygote advantage at allozyme loci (Bierne *et al*., 2000). Therefore, the observed fitness differences are attributed to the loci linked to the allozyme loci which are considered to be selectively neutral (Mitton, 1998). One argument opposed to this view is that the loci in linkage disequilibrium with the allozyme loci that are assumed to be neutral can not be maintained in the absence of selection for long evolutionary periods (Clarke, 1970). The effect of the associative overdominance on allozyme loci depends on the strength of the linkage disequilibrium between the selected and the allozyme loci, and it particularly influence populations with small size where organisms have low rates of genomic recombination events. (Thelen and Allendorf, 2001).

Several studies have been performed to test the associative overdominance hypothesis by comparing the consequences of heterozygosity at allozyme and DNA markers such as microsatellites and RFLPs on the fitness of the organisms (Thelen and Allendorf, 2001; Borrell *et al*., 2004). For example, the examination of the association between heterozygosity at genetic markers (six allozyme and eight microsatellite loci), and fluctuating asymmetry, length and weight in Atlantic salmon (*Salmo salar* L.) has revealed that heterozygosity at allozyme loci, not the microsatelites contributes to the increased fitness of the organisms (Borrell *et al*., 2004). Furthermore, Thelen and Allendorf (2001) reported that individuals with high level of heterozygosity at allozyme loci had a significantly higher fitness, however such relationship was not observed for the heterozygosity at microsatellite loci. Considering honeybees it has been determined that honeybees have significantly higher levels of recombination events per choromosome during meisosis and the unusually high recombination rate in honeybee genome is thought to contribute to efficient removal of the deleterious mutations (Beye *et al*., 2006)

1.5. Biology of the Honey bees

Like ants and wasps bees are social insects, living in communities with highly developed age related, and socially regulated division of labor (Robinson, 2002). Bees living in such social communities are classified under the Apinae subfamily of Apidae family. Apinae subfamily includes ten species of honeybees: *Apis dorsata*, *Apis binghami*, *Apis laboriosa, Apis andreniformis, Apis florea, Apis mellifera, Apis cerana, Apis koschevnikovi, Apis nuluensis, and Apis nigrocincta* (Sheppard and Arias, 2005).

Honeybees are of both ecological and economical importance because while collecting pollen and nectar in order to maintain their survival, they at the same time contribute to the pollination of plants. About 80 % of agriculturally important crops are pollinated by animal pollinators most of which are bees (FAO, 1980).

1.5.1. Hive members and Social Life

Honeybees live in colonies usually containing on average 20 000 individuals (Nerum and Buelens, 1997). Inside the hive there are three casts: queen, is the only egg lying female and all the members of the colony; males or drones and workers are her offspring. Caste determination is independent of genetic differences and occurs through differences in larval nutrition (Winston, 1987). Larvae fed with nutrient-rich royal jelly become queens, whereas larvae that are fed with a less rich diet become workers. The queen usually mates only once in her life with 12-15 drones and stores the sperms received from the drones in a sperm sac in her abdomen. The egg laying behavior of the queen is affected by environmental conditions and there are seasonal differences in the egg laying rate: in winter very few eggs are produced whereas in summer she can lay up to 2000 eggs per day (Bodenheimer, 1937). Fertilized eggs give rise to female worker bees in the colony that perform all the tasks required for the survival and maintenance of the colony. Drones develop from unfertilized eggs and their main function is to fertilize the queen. This type of reproduction is common to hymenoptera in which workers and queens are diploid (2n), whereas males are haploid (n) accordingly, these animals are named as haplodiploid organisms.

Social structure of a colony is maintained by complex mechanisms of interactions and communication among the members of the hive (Robinson, 1999). Pheromones are important components of the social life that enable the bees to gather and integrate information regarding the colony needs and environmental conditions (Wilson, 1965; Le Conte and Hefetz, 2008) and as molecules of communication they play important roles in processes such as inhibition of the ovary development in workers to maintain reproductive division of labor (Mohammedi *et al*., 1998), in defensive behavior against a potential enemy (Nunez *et al*., 1998) and regulation of behavioral development and transitions (Le Conte *et al*., 2001; Leoncini *et al*., 2004).

Primer pheromones are found to be important determinants of interaction and communication molecules whitin a colony that have significantly influence the social structure and division of labor among worker bees (Le Conte and Hefetz, 2008). For instance, as reported by Pankiw *et al*. (1998) nurse bees that were exposed to queen mandibular pheromone (QMP) increased the duration of the nursing period and transition to foraging was delayed in response to application of (QMP). Le Conte *et al*., (2001) reported that also the brood pheromone leads to foraging at older ages and depresses the juvenile hormone titers. Furthermore, Ben-Shahar *et al*., (2002; 2003) demonstrated the role of cGMP-dependent kinase, PKG which is encoded by the foraging gene, *Amfor,* in transition from nursing to foraging. Forager bees have higher PKG activity levels. And a recent work by Whitfield *et al.*, (2006) conducted microarray analyses and provided evidence for multifactorial genetic regulation of complex social behavior in interaction with environmental information. As these studies suggest, communication between colony members, social structure and the behavioral development in honeybee colonies are all related with each other in complex mechanisms that are affected and regulated by the genetic, environmental and intracolonial factors (Giray *et al*., 1999, 2007).

Worker bees in the colony can control the amount of brood by regulating the number of cells they prepare or thorough cannibalism of larvae (Schmickl and Crailsheim, 2001). Cannibalism behavior of the workers is significantly affected by both environmental factors such as pollen and nectar availability, weather conditions, and also by the conditions in the colony (Schmickl and Crailsheim, 2002). Each larva develops in individual open cells. Worker bees called nurses produce a fluid substance named jelly and feed the larvae. The ingredients of the jelly are mainly produced from pollen (Moritz and Crailsheim, 1987). Honey and nectar are the main carbohydrate sources within a colony and they are in high amount in contrast to pollen which is the main protein source (Schmickl and Crailsheim, 2002). The amount of brood and the brood rearing efficiency is primarily determined by the amount of pollen in the hive, the ratio of the amount of pollen to the number of larvae has a significant effect on brood nursing (Schmickl and Crailsheim, 2002; Matilla and Otis, 2007).

Nursing the larvae is not the only task of the workers. Socially regulated and age-dependent polyethism means that workers within a hive pass through behavioral transitions that determine their task in the hive in an age related pattern (Winston, 1987; Page and Peng, 2001). Following the emergence of workers as winged adults after completing 21 days of egg, larval and pupal stages they perform a variety of activities that is required for the maintenance of the colony. A newly emerged bee generally consume high amount of pollen and may clean empty cells so that the queen can lay eggs and in the next 3-14 days she starts synthesis of jelly to feed the larvae and the queen and use wax to prepare cells, thus become a nurse bee (Toth and Robinson, 2005). Within the 15-20 day period in addition to comb building and nectar processing workers become guarders that defense the colony against foreign insects and undertakers who carry the dead bees away from the hive. After 2-3 weeks the workers start foraging for pollen, nectar and water outside the hive which involves a high expenditure of energy (Huang and Robinson, 1992).

The physiology of the bees also change as they age and switch from working inside the hive to forage outside; the juvenile hormone levels, water content of the body, thorax glycogen content, metabolic rate and flight capacity increase and body mass decreases (Harrison, 1986; Huang *et al*., 1994; Giray *et al*., 1999; Roberts and Elekonich, 2005). Age related division of labor and the behavioral development of honeybees are highly flexible and responsive to the social and physical environment (Robinson, 1999, 2002). Depending on the needs of the colony and environmental conditions bees can accelerate, delay or reverse their behavioral developmental transitions (Robinson *et al*., 1992; Giray and Robinson, 1994).

For instance, when the number of nurses within the colony is less than required, nurse bees continue to feed the larvae rather than transition to tasks outside the hive (Robinson *et al*., 1989, 1992; Giray and Robinson, 1994; Roberts and Elekonich, 2005). Furthermore, it has been demonstrated that the adult behavioral development of honeybees also differs depending on the genetic background of the colony and responsive to factors such as season, weather, nutritional status (Schulz and

Robinson, 1999) and parasites (Page *et al*., 1992; Huang and Robinson 1995; Giray *et al*., 1999). Behavioral development is accompanied by the biochemical and physiological changes that produced by the activity of various enzymes in different tissues (Roberts and Elekonich, 2005). The fact that the age demography of the colony, metabolism, physiology and activities of the bees differ in winter and summer, raises the question of how these seasonal changes are associated with enzyme loci.

1.5.2. Honeybees in winter and summer

Due to seasonal fluctuations in temperature and because of the wide range of geographical distribution honeybees encounter a wide range of temperatures from sub zero values up to values higher than 40°C (Heinrich, 1993). In summer and winter honeybee colonies show various differences in terms of behavior, physiology, population structure and age demography (Bodenheimer, 1937; Merz *et al*., 1979; Seeley and Visscher, 1985). Each of these metabolic changes contributes to the survival of honeybees in winter.

With the arrival of the winter, a colony changes its population structure by reducing the amount of brood in the colony and shifting from an active summer colony to a hibernating winter colony (Matilla and Otis, 2007). Analysis of the factors that regulate the reduction in brood amount by transition from summer to winter have revealed that, in addition to changes in temperature and photoperiod with the approach of winter, availability of the pollen sources is significantly effective on both the time of initiation of the decrease in brood-rearing activity and the timing of the appearance of long-lived workers in colonies because it has been observed that the colonies with extended pollen supply produced more workers during autumn than the colonies with less pollen (Matilla and Otis, 2007).

In summer the number of the bees in the hive can rise up to 70 000 to 100 000 consisting of short lived (life span around 30 days) individuals and the temperature inside the nest is approximately 34-35°C (Nerum and Buelens, 1997). Whereas, in

winter, during hibernation the number of the colony members reduces down to 10 000 to 20 000 consisting of long living (life span can be several months) bees (Matilla and Otis, 2007). Although very little brood can be reared in winter, colony can survive due to the longer life span of the winter bees (Fukuda and Sekuguchi, 1966; Matilla and Otis, 2007).

It has been determined that the life span of worker honeybees is depends on the duration of the hive period vs. the foraging period which is characterized by both the duration and total flight performance during foraging (Neukirch, 1982). This finding may explain the longer life span of the winter bees remaining inside the hive throughout the cold winter period. Winter bees do not perform foraging flights as they do in summer; however in winter all workers require energy to produce heat and maintain high temperatures in the hive. They form clusters in which temperatures range from 12°C in the surface and to 13-35°C in the core (Johansson *et al*., 1979; Stabentheiner *et al*., 2002). Bees in the winter cluster maintain such a temperature range in the hive by the constriction of their thoracic muscles (Beenakkers *et al*., 1984). Winter cluster is generally formed by bees when the temperature falls to 15°C (Southwick, 1991). This social thermoregulation enables the bees to survive in the cold temperatures of the winter (Heinrich and Esch, 1994; Panzenbock and Crailsheim, 1997). The mechanism of thermoregulation relies on the release of heat energy as a result of the breakdown of the carbohydrate sources by the worker bees in the cluster (Stabentheiner *et al*., 2002). It has been reported that heating of the colony by thermoregulation of the bees consumes approximately the same amount of metabolic energy as the foraging of bees in summer and consequently the metabolic state and glycogen stores of heating bees (bees in cluster) and the foragers are similar (Panzenbock and Crailsheim, 1997).

In addition to the temperature variation in the cluster (surface vs. core temperature differences) honeybees show variations in their glycogen content depending on their position in the cluster whether they are in the core or on the surface; bees that are near the surface of the cluster have significantly higher glycogen reserves compared to the bees in the core of the cluster; there is a gradient of glycogen reserves from

high to low moving from the surface to the core of the cluster (Panzenbock and Crailsheim, 1997). This variation in glycogen content can be due to the expenditure of energy for thermoregulation since it has been demonstrated that bees in the cluster (especially those in the core region) increase their metabolic rate to produce heat and therefore increase their food consumption (Free, 1957; Panzenbock and Crailsheim, 1997). In summer, honeybees have been determined to have lower glycogen reserves as compared to the winter (Panzenbock and Crailsheim, 1997). Therefore, especially in winter, the glycogen reserves of bees may become an important factor that contribute to the social thermoregulation of the nest which is a unique adaptation to maintain survival in the challenge of winter.

1.6. The use of Allozymes in measuring genetic variation and identification of population structure

Allozyme electrophoresis is a useful biochemical method that is extensively used in determination of the genetic variation in natural populations. The term "allozyme" (allelic isozymes) is used to denote the different allelic forms of DNA encoded enzymes, whereas "isozyme" is the term that refers to the different biochemical forms of functionally similar enzymes encoded by distinct but related loci (Prakash *et al*., 1969; Buth and Murphy, 1998).

Allozymes are co-dominant molecular markers that allow the identification of allelic composition of the homozygote and heterozygote individuals which in turn can be used to estimate and evaluate the genetic structure of the studied population by calculations of degree of heterozygosity, gene diversity, gene flow, degree of polymorphism at the loci examined and other statistical parameters. The observed genotype frequencies can be compared with the Hardy-Weinberg expectations to evaluate the consequences of deviations. A particular genetic locus is said to be monomorphic if a single allele predominates with a proportion of 99% or more of the population or polymorphic if it expresses more than one allele in 99% of the

population (Micales and Bonde, 1995). The identification of the genotypes is performed by determining the allelic composition of each individual sampled for the study. The number of alleles observed depends on both the genetic structure of the organism and the enzyme loci studied. The nuclear condition of the organism whether it is monokaryotic or dikaryotic, ploidy number (haploid, diploid, polyploid), and genetic makeup (homozygous, heterozygous) of the organism together with the quaternary structure of the enzyme determine the number of alleles observed on the gel (Micales and Bonde, 1995).

In addition to these factors, the results obtained from an electrophoretic study can significantly differ depending on the presence of post-translational modifications, variations in photoperiod and diet of the organism, sample processing procedures, sample storage time, and experimental methodology (Poly, 1997). Phosphorylation, deamination and acetylation are among the common post-translational modifications that may lead to differences in the electrophoretic mobility and several procedures are available to remove the associated group from the enzyme; for example carbohydrates that are attached to proteins forming glycoproteins can be treated with the enzyme that degrades the carbohydrate unit (Micales and Bonde, 1995). Also it is important to consider the environmental variations such as diet, photoperiod, salinity and temperature since they may affect the expression patterns of the enzymes which in turn lead to the observation of different banding patterns of allozymes (Poly, 1997). Furthermore, the expression of various allozymes was found to differ both temporally depending on developmental processes and also spatially depending on the tissue (Micales and Bonde, 1995). Such factors may interfere with the results and may lead to erroneous conclusions. Therefore, it is important to standardize both the sampling of the organisms considering factors such as age, sex, tissue used in the analysis and also environmental variables together with the experimental method by using several buffer systems with different substrate and cofactor concentrations to optimize the electrophoretic study and to avoid arriving erroneous results.

1.6.1. Temporal Changes in Allozyme Genotype Frequencies

Many allozyme studies that is performed for the characterization of genetic variation in natural populations have analyzed variations at enzyme loci and compared the differences in the genetic structure of allozymes from different geographical regions assuming that the protein variation is temporally constant and therefore can be compared geographically (Moffett and Crozier, 1996). The inaccuracy of this assumption is supported by evidences accumulating as a result of the studies that focus on the temporal changes in allele and genotype frequencies at enzyme loci (Rank and Dahlhoff, 2002; Ward *et al*., 2004; Martinez *et al*., 2005). Cyclical shifts in allele and genotype frequencies of enzymes may enable the organisms to adapt to temporally changing environmental variations such as seasonal fluctuations in temperature (Rank and Dahlhoff, 2002). Ward *et al*. (2004) studied the seasonal variation in *Pgm* allozyme frequency in a yellow dung fly, *Scathophaga stercoraria* population and found a relationship between the frequency of the most common *Pgm* allele and the temperature it is being the least frequent in summer and at intermediate temperatures. In *S. stercoraria* it has been shown that *Pgm* genotypes differ in larval growth rates under different environmental conditions and produce adults with different sizes (Ward 1998), and the females of this species have been shown to choose males of different *Pgm* genotypes under different environmental conditions (Ward, 2000). In addition to the seasonal changes in genotype frequencies of allozymes, it has been documented that such cyclic changes can occur also at the chromosome level. A whole year, monthly analysis of enzyme and chromosome polymorphisms resulting from inversions in *Drosophila pseudoobscura* and *Drosophila persimilis* have revealed that changes in the genotype frequencies follow seasonal patterns (Dobzhansky and Ayala, 1973).

Therefore, it is evident that seasonal variations exist at enzyme loci and there is also temporal variation for the allozymes of the organisms collected from the same location. Analysis of genetic variation in natural populations of species should take

into account that in addition to the geographical location of sampling, the time of the year when sample collection is done may change the conclusions of allozyme analysis.

Seasonal changes in allele frequencies have generally been associated with the changes in temperature. As an explanation to the temperature dependent changes in allozyme frequencies, it has been suggested that such shifts occur as a result of differential gene expression in response to variations in temperature (Hochachka, 1967; Somero, 2004). Accordingly, it has been suggested that the significant variations in the concentrations of enzymes are due to the variations in expressions of the regulatory genes which may have more significant roles in affecting the fitness of the organism than the variations in the biochemical and catalytic properties of the enzymes (Allendorf *et al*., 1982). This view has been supported by numerous authors who have emphasized the significance of epigenetic effects on enzyme loci and proposed that genetic variations in the regulatory genes may have more evolutionary significance than the genetic variations at enzyme loci (Wilson, 1976; Ayala and McDonald, 1981; Allendorf *et al*., 1982).

In identifying the "strategies" by which enzymes adapt to changes in temperature, Somero (2004) has summarized the main mechanisms in three categories as: 1) changes in the primary structure (amino acid sequence) of the enzyme that lead to adaptive variation in catalytic properties and thermostability parameters, 2) changes in the cellular concentrations of the enzyme which are caused by changes in the expression of the gene that code for the enzyme, 3) changes in the "milieu" in which enzymes perform their activities. Development of the molecular techniques that enable the determination of the gene expression profiles and sequencing of amino acid and DNA fragments has facilitated the identification of the mechanisms by which amino acid polymorphisms lead to significant variation in metabolic activity.

Analysis of enzyme variation over an extended period of time may provide clues about the physiological changes that occur in response to the changes in climatic

changes which in turn can assist in the identification of the molecular mechanisms that maintain the high amount of genetic variation in natural populations.

In previous studies that were performed for the determination of genetic variation in honeybee subspecies in Turkey, it has been observed that the amount of genetic variation in honeybee populations in Turkey is significantly higher than those observed in other regions in the world (Kandemir *et al*., 2000). Furthermore, *Pgm* locus has been observed to have the highest amount of polymorphism among all the loci studied. One of the remarkable outcomes of these studies has been the observation that in two southeastern provinces, Hatay and Gaziantep honeybee populations, *Pgm* heterozygotes were found at higher frequencies in winter (Hadimoğulları *et al*., 2002). Although the results obtained from this study indicate the seasonal variation of allele and genotype frequencies at *Pgm* locus in honeybees, a whole year survey of enzyme polymorphism has not been analyzed by regular sampling periods to monitor the change in gene and genotype frequencies in different regions and subspecies.

1.7. The Objective of the Study

Main objectives of this study are:

- A whole year analysis of allozyme variation at *Pgm* and *Hk* loci of honeybees by regular sampling in each month to identify and characterize the mode of seasonal variation of three subspecies namely *A.m. carcica*, *A.m. caucasica* and *A.m. syriaca* from three ecologically different locations; Kırklareli in Thrace, Artvin in northeastern Black sea, and Hatay in southeastern Anatolia, respectively.

- Correlation of different *Pgm* genotypes with PGM enzyme activity and glycogen content to evaluate the biochemical and physiological consequences of the seasonal variation in *Pgm* genotype frequencies

- Determination of the relationship between shifts in *Pgm* genotype frequency and changes in temperature to understand the mechanisms by which honeybees respond to these seasonal temperature changes.

- Evaluation of the reasons for the seasonal variation in of *Pgm* heterozygosity in order to understand this interesting phenomenon in honey bees.

CHAPTER 2

MATERIALS AND METHOD

2.1. Biological materials

2.1.1. Samples in allozyme analysis

The total number of worker bees studied in the allozyme analysis is 2540 (Table 2.1). They were collected and put in small plastic bottles, labeled and fed with Turkish Delight (water+saccharose+starch) and brought alive to the laboratory (Kandemir *et al*., 2000). Dead bees and drones (since drones are haploid, no heterozygosity can be observed) were discarded. Samples were labeled with date and collection site and stored at -80 until homogenization.

2.1.2. Samples in enzyme activity assays, glycogen and protein measurements

Fresh samples were used for PGM enzyme activity assays to avoid any decrease in enzyme activity. Assays were conducted with four distinct pools of 10 (total number of 40) individual bees collected in summer and winter months. Glycogen content and soluble protein measurements were performed with the same samples that were used in the analysis of enzyme activity.

2.1.3. Chemicals used in the analyses

All chemicals were purchased from Sigma chemical company, US. The list of the chemicals used in all analyses is given in Appendix A.

2.2. Methods

2.2.1. Sampling

In order to make a valid inference about a population we need to sample in such a way that it reflects the variation in the natural population. Since the honey bee (*Apis mellifera* L.) workers of individual colonies are generally descended from a single queen, extensive sampling of a low number of colonies from a single location provides information about only a few genomes which can not provide informative data about the population. Multiple bees collected from the same colony over represent the alleles of the queen. Instead, sampling of a few workers from each of several colonies is more suitable.

Five worker bees were collected each month from each of ten apiaries from Kırklareli, four bees from Artvin and Hatay provinces in order to examine the seasonal change in allozyme frequencies. Sample collection times, number of bees studied per month and the total number of bees studied throughout the electrophoretic analysis is given in Table 2.1.

Enzyme activity assays, glycogen measurements and total protein measurements are performed by using four distinct pools of 10 individual bees.

Table 2.1. Areas of sample collection with corresponding sample size and sample collection interval

Sample Collection Location	Name of the subspecies	Sample Collection Time	Sample Size / Month
NORTHWESTERN ANATOLIA	*A.mellifera carnica*	October 2005-October 2007 (26 months)	**50**
NORTHEASTERN ANATOLIA	*A.mellifera caucasica*	July 2006- November 2007 (17 months)	**40**
SOUTHERN ANATOLIA	*A.mellifera syriaca*	Nov. 2006- Nov. 2007 (14 months)	**40**
	TOTAL		**2540**

Figure 2.1. Geographic location of the sample collection areas.

The locations where the sampling of the bees were made exhibit extensive ecological differences, extending from near subarctic conditions with up to 9 months of snow cover (Artvin) to temperate rain forests to subtropical regions with 12 months of warm weather (Hatay) and to a more temperate climate (Kırklareli).

A.m. carnica (samples collected from Kırklareli) is found in the Thrace region (border with Bulgaria). This region has temperate climate with four seasons. The area has dry summers but has moderate precipitation at other times. The plant cover is of two types; deciduous forest and bushy vegetation. The Kirklareli region within Thrace has been proposed by Kence and Kence for protection of the *A.m. carnica* to Turkish Ministry of Agriculture.

A.m. caucasica is (samples collected from Artvin) found in eastern Turkey (border with Georgia). In this area conditions are similar to subarctic climate with harsh winters and up to 8 months of snow cover from October through early June. This area is a biosphere region announced by World Wildlife Fund, and it is under protection by Turkish Ministry of Environment and Forestry. Migratory beekeepers are not allowed to this region.

A.m. syriaca (samples collected from Hatay) populations are present in southeastern Turkey, in areas bordering Syria. This region has a subtropical mediterranean climate with warm temperatures throughout the year and high precipitation in winter.

2.2.2. Electrophoretic Analysis of Allozyme Variation

2.2.2.1. Preparation of the homogenates

Frozen honeybees were dissected, thoraces of honeybees were homogenized with a motorized grinder in 300µl distilled water and centrifuged for 10 minutes at 12000 rpm, supernatants were transferred in microtubes stored in – 80 °C until used.

In preliminary experiments during optimization, comparison of activity of samples homogenized in 300µl of Tris-HCl, pH 7.0 grinding buffer and 300µl distilled water resulted in the observation that the use of grinding buffer did not affect the enzyme activity but it resulted in poor color development in glycogen assays, due to interference of the assay with the components of the buffer. When all samples are homogenized in distilled water, all assays were performed without interference. Homogenizations of the samples were performed on ice to prevent an increase in temperature. It is important to prevent the denaturation of enzymes during sample preparation.

2.2.2.2. Electrophoresis

The identification of different forms of enzymes by the process of electrophoresis is one of the most useful research tools in the analysis and characterization of genetic variation in natural populations. Electrophoresis is the movement of charged molecules in a buffered gel, under the influence of an electric current. Charged molecules and particles migrate in the direction of the electrode bearing the opposite charge when electric current is applied. Because of the variation in their charges and masses, different molecules will migrate at different velocities which in turn results in the separation of these molecules according to their electrophoretic mobility. Electrophoretic mobility is a significant and characteristic parameter of a charged molecule and can be influenced by several factors including type, concentration and pH of the buffer, the field strength, temperature, concentration and the nature of the

support material that forms the gel. Different enzymes and the alternative forms of similar enzymes migrate at different rates when current is applied to samples in a buffered starch gel.

After the application of the electric current to the gel for a sufficient time, gel slabs are sliced and can be stained for different enzymes by placing gel slices into a dye solution that contains the substrate and cofactor specific to the enzyme studied, along with a dye that precipitates where the enzyme catalyzed reaction occurs (Soltis *et al*., 1983). As a result, reaction products can be visualized as distinct dark bands on the gel. The locations of visible bands on the gels mark the migration distances of specific enzymes (Murphy, 1993). In staining of the gels, the reaction taking place can be symbolized in the following way as shown in Figure 2.2:

Figure 2.2. Symbolic representation of the reaction that occur during histochemical staining

In this study, thin layer native horizontal starch gel electrophoresis was used. The basic laboratory setup for electrophoresis is illustrated schematically in Figure 2.3.

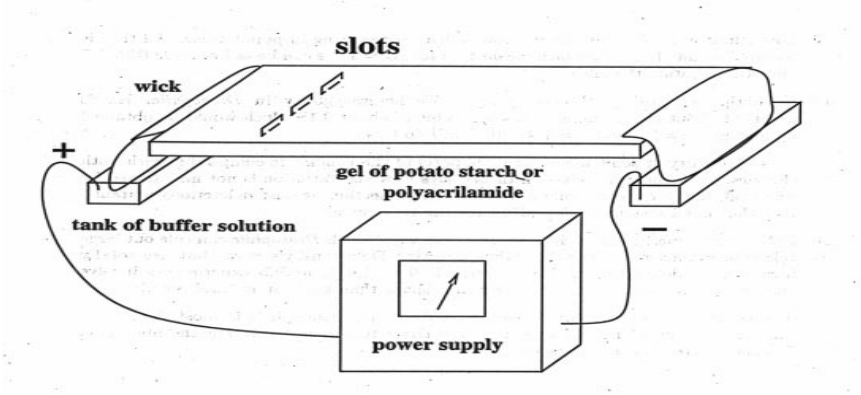

Figure 2.3. The basic setup for horizontal starch gel electrophoresis equipment

2.2.2.3. Preparation of the Starch Gel

When starch is cooked within a buffer solution suitable for the enzyme studied and cooled enough, forms a gel. The concentration of starch in the buffer solution determines the consistency of the gel (Conkle *et al*, 1982). The concentration of the starch can be adjusted to obtain gels with the required characteristics that work best depending on the properties of the enzyme studied. Major variables that may affect the quality of the starch gel are brand of starch, the quality of distilled or deionized water, starch lot, laboratory and refrigerator temperatures, and the length of time that gels are vacuum-degassed (Conkle *et al*., 1982).

It is important to maintain a constant starch concentration throughout a study for comparison of banding patterns between gels. In this study a variety of starch concentrations have been experimented in the range of 10.5 to 14% and the concentration of 12% was found to be most suitable for PGM and HK and this concentration is maintained throughout the study in the analysis of both enzymes.

- 36 gr starch was weighed, together with the 300 ml gel buffer in to the flask. Flask is swirled over a flame until the gel thickens. (It is important to swirl the mixture continuously to keep the starch well suspended, then vigorously shaked side by side to prevent scorching, until the gel gets thinner and clearer)

- Then the gel was degassed with a vacuum pump for 15-20 seconds until small bubbles (dissolved air) disappear and large bubbles (water vapor) appear. Slow swirling of the flask while degassing is important to maintain consistency of the gel. The process of degassing should last only 15 to 20 seconds; the starch may polymerize inside the flask if degassing is prolonged.

- After degassing, the gel was poured into the gel mold. Globs and air bubbles were picked out if any.

- The gel mold was put in to the refrigerator to fasten polymerization. After 15 to 20 minutes, when the gel has gone from transparent to opaque, the upper layer of the gel mold was sliced before loading the samples for better resolution (staining becomes too difficult if gels are sliced after loading the samples).

2.2.2.4. Loading and Running Starch Gels

For continuous buffer systems (those in which the gel and the electrode buffers are the same except for concentration) a long gel can be poured and made 2 or 3 sample slices one near one end and the others near the middle after slicing the upper layer of the gel (McDonald, 1985). This method is termed as *piggybacking* a gel and it is useful when the sample size is high since saves both the money and time especially when multiple enzyme loci are analyzed.

Loading of the samples to the gel was performed on ice and all the homogenates were kept in ice to avoid any decrease in activity. For loading samples, paper wicks (Whatman filter paper) were dipped into the homogenates to absorb the mixture of proteins from the homogenized samples and put into the sample slots. To allow comparison of the genotypes on different sample slots, the last sample loaded to the first slot is reloaded to the second slot. When all samples were loaded two paper wicks were dipped into Bromophenol Blue solution also added as a marker dye front on both sides of the sample slots to monitor the progress of the gels as long as the current is on. 250 ml of refrigerated electrode buffer (Table 2.2) was poured into each electrode tray and the gel was placed on the cooling plate of the gel tank, the sponge wicks were placed on both ends of the gel to cover about 1 cm of plastic and all the exposed gel surface, and extended down into the tray to contact electrode buffer. Sponges were completely saturated with electrode buffer to prevent drying

and pressed against the gel to make complete contact. Before running the voltage and the current of the power supply was adjusted to keep current constant during. The cooling unit of the electrophoresis tank maintained the temperature of the ceramic plate on which the gel was placed constant at 4 °C throughout the run to prevent warming and denaturation of the enzymes during the run.

The most suitable electrophoresis system and running conditions are given for the 2 enzymes (Phosphoglucomutase- PGM, Hexokinase- HK) studied in Table 2.2.

Table 2.2. Electrophoretic conditions for PGM and HK.

Enzyme	**Buffer System**	**Running Temperature**	**Voltage & Current**	**Running Time**	**References**
PGM	Tris-Maleic pH. 7.4	4 °C	170 V 30mA	5hrs	McDonald, 1985 Shaw and Prasad, 1970
HK	Tris-Citrate pH. 8.5	4 °C	150 V 15mA	5hrs	Badino *et al*., 1983

2.2.2.5. Staining the Starch-Gels

When the electrophoresis is complete, the next step is the qualitative or quantitative analysis of the gel which is possible only after the staining procedure since the products of the enzyme reactions are not visible or colored at the end of the electrophoresis. The specific histochemical staining of the starch gel enables determination of the location and amount of the separated molecules.

The genotypes of the loaded samples and the alleles of the genotypes can be visualized after histochemical staining which consists of an enzyme-specific substrate, the necessary cofactor and oxidized salt that link the enzyme and results in a dye precipitate (Conkle *et al*, 1982). This method is also named as *Activity Staining* since the success of the staining step depends on the maintenance of the activity of the proteins throughout the electrophoretic run.

Two different methods are present for the staining of starch gels. The first one is the *Direct Staining* in which the filter paper is put on to the gel and the stain is poured onto the paper. Any air bubbles or wrinkles are smoothed in the paper. In this method the gel is not put into an incubator since it can cause drying of the gel, and there is no need to keep the gel in the dark conditions.

In this study we used *Agar Overlay Staining* method (McDonald, 1985), in which equal volume of agar solution was added to the stain solution and poured on to the gel. Then, when the stain was firm, it was put in a dark 37°C incubator. Dark conditions are required since stains containing PMS and MTT turn blue after 15-20 minutes or so in the light. The staining systems for the studied enzymes are given in Table 2.3. If some ingredient of the staining solution is forgotten the agar layer can be scraped carefully off the gel and can be stained again. Agar overlay staining method also allows the transfer of the results to chromatography paper which can be maintained permanently.

Table 2.3. Staining systems for the enzymes studied.

Enzyme	Stain Ingredients
PGM	Tris-HCl pH. 8.0 staining buffer Glucose-1-Phosphate NADP $MgCl_2$ Glucose-6-Phosphate Dehydrogenase PMS MTT Agar Solution
HK	Tris-HCl pH. 8.0 staining buffer Glucose ATP $MgCl_2$ NADP Glucose-6-Phosphate Dehydrogenase PMS MTT Agar Solution

2.2.2.6. Fixation and documentation of the gel

In order to keep a permanent record of results, we routinely photographed gel slices using gel visualization system. After taking the photograph, the agar layer on the gel was taken on to a chromatography paper, Whatman and fixed in 3M acetic acid.

2.2.2.7. Interpretation of the Starch Gels

After staining, the gels are examined and the genotype of each sample is recorded. The allelic forms of the enzymes that can be visualized on a gel are called electromorphs. Interpretation of the electromorphs on a gel requires understanding of the genetic basis of allelic variation and structural properties for each enzyme.

The primary structure of an enzyme; the sequence of its amino acids is determined by the specific sequence of the gene which codes that enzyme; therefore variations in

proteins indicate genetic variation. Some amino acids in the structure of a protein carry a net negative or positive charge and some are neutral. Therefore, a protein has a net charge, which is the sum of the charges of its constituent amino acids. If a variant protein (caused by a variant DNA sequence) has an amino acid substitution that changes the net charge of the protein that variant protein will behave differently under the influence of an electric field. The change in the overall electric charge of the protein will become detectable due to the change in electrophoretic mobility.

In addition to the variations in the primary structure, the observed electromorphs provides information regarding the quaternary structure of the enzyme. Homozygotes at a given locus typically yield one band, while the heterozygotes typically yield two, three or five bands, depending on the quaternary structure of the enzyme; the number of the subunits in the structure of the enzyme. If an enzyme is monomeric (consists of a single polypeptide unit), each different band position results directly from a different polypeptide coded by a different allele. When there are two or more alleles in a population, a heterozygote will possess two bands, and a homozygote will possess a single band. If an enzyme is dimeric (consisting of two polypeptide units), the different band positions reflect the different combinations of polypeptide units. If there are two different alleles in an individual, there will be three bands because there are three possible combinations of the polypeptide subunits coded for by the alleles. Typical banding patterns of a monomeric and a dimeric enzyme coded by a single locus are illustrated in Figure 2.4.

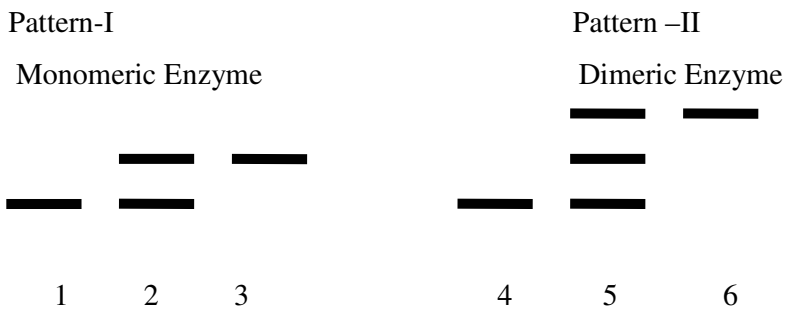

Figure 2.4. An illustration of the banding patterns of a monomeric and a dimeric enzyme (sample 1,3,4,6 illustrate homozygote individuals, sample 2, 5 illustrate heterozygote individuals).

2.2.2.8. Studied Enzymes

2.2.2.8.1. Phosphoglucomutase (PGM) E.C. 5.4.2.2 formerly E.C. 2.5.7.11

Phosphoglucomutase (PGM) is a key enzyme in the carbohydrate metabolism of variety organisms catalyzing an important regulatory point in carbohydrate metabolism, the reversible transfer of a phosphate group between Glucose-1-Phosphate and Glucose-6-Phosphate. The phosphorylated sugars may enter catabolic pathways, to produce energy or enter anabolic pathways, leading to the synthesis of polysaccharides mainly glycogen in animal tissues and starch and cell wall polymers in plants. PGM enzyme is composed of a single subunit (monomeric in structure) and mainly found in cytoplasm of all cells.

$$\text{Glucose-1- Phosphate} \xleftrightarrow[Mg^{+2}]{PGM} \text{Glucose-6-Phosphate} \qquad (2.1)$$

2.2.2.8.2. Hexokinase (HK) E.C.2.7.1.1

HK is one of the most widely distributed enzymes, monomeric in structure and mainly found in the cytoplasm of most cells. The first reaction in the glycolysis is the transfer of a phosphoryl group from ATP molecule to glucose to form Glucose-6-Phosphate in a reaction catalyzed by hexokinase (HK). But mechanism of action of HK is not as specific as PGM since HK catalyzes the phosphorilation not only of D-glucose but also a variety of other hexoses and hexose derivatives such as D-fructose, D-mannose and D-glucoseamine; it has a higher affinity for aldohexoses than for ketohexoses (Lehninger, 1982).

$$\text{ATP}+\alpha\text{-D- Glucose} \xrightarrow[Mg^{+2}]{HK} \alpha\text{-D- Glucose-Phosphate}+\text{ADP}+ H^{+} \quad (2.2)$$

2.2.3. Spectrophotometric Analysis of Enzyme Activity, Glycogen and Protein concentration

2.2.3.1. General Information about Spectrophotometric Measurements

Spectrophotometric techniques are used widely to study the structure and reactions of compounds both quantitatively and qualitatively. An important advantage of these techniques that makes them useful in biological studies is that they do not degrade

the molecules analyzed (Bradley and Markwell, 2007). In addition, these techniques enable the analysis of very small amounth of material even if the studied material is found systems where many other molecules are present (Lehninger, 1982).

Every compound has a specific spectrophotometric absorption, like a "fingerprint", that allows its identification and characterization usually, in the presence of other compounds (Lehninger, 1982).It is also possible to determine quantitatively the concentration of that compound by spectrophotometric assays. The relationship between absorbance and concentration is explained by the Beer-Lambert Law and is written mathematically as:

$$A = \varepsilon.c.d$$

A =Absorbance (no units)

ε = molar extinction coefficient for the absorbing material at the wavelength λ (in units of $dm^3\ mol^{-1}\ cm^{-1}$)

c = molar concentration of the absorbing solution

d = light path in the absorbing material in centimeters (2.3)

Thus, according to the equation, the Beer-Lambert Law states that the concentration of a substance is proportional to the amount of light that it absorbs (Lehninger, 1982).

In the enzyme activity measurements, the assay solution contains many other compounds that are required for the activity of the enzyme to be studied. These compounds in the reaction mix may absorb the same wavelengths as the enzyme under study. To get rid of this potential source of interference of other compounds, the absorbance of test solution is compared to a specific blank solution. The blank contains everything found in the sample solution except the substance to be analyzed or measured. This enables the subtraction of the effect of the compounds that are not measured in the assay.

2.2.3.2. Determination of Enzyme Activity

Enzyme activity assays were conducted with four distinct pools of 10 (total number of 40) individual bees collected in summer and winter months. The individual bees used in activity assays were chosen from the freshest (latest collected) samples.

Frozen honeybees were dissected, thoraces of honeybees were homogenized with a motorized grinder in 300µl distilled water and centrifuged for 15 minutes at 12000 rpm (the same procedure as in the preparation of homogenates for electrophoretic analysis but the centrifugation time was longer to prevent the contamination of cellular debris) , the supernatants were removed and immediately placed on ice. PGM activity assays were completed in the same day that the homogenization of samples was done. The remaining homogenates were frozen at -80ºC for the glycogen and protein measurement assays and electrophoretic analysis.

2.2.3.2.1. Procedure for PGM Activity Assays

Assays for PGM activity were carried out on a Shimatzu UV/visible spectrophotometer equipped with a temperature-controlled cuvette holder at 25ºC by the optimization of the method used by Verrelli and Eanes (2001b). The assay reaction mix was prepared freshly (immediately before performing the assay) on the same day that the activity measurements were performed. The composition of the PGM activity assay mix is as follows:

0.85mM G-1-P

0.5mM NADP $\rightarrow\rightarrow\rightarrow$ dissolved in 20mM Tris-HCl (pH, 7.4)

1.0mM $MgCl_2$

3.2 units/ml G-6-P-D

The assay contained 945µl of the reaction mix and 55µl of homogenate (a total volume of 1 ml).

- The spectrophotometer was turned on to allow it to warm up for at least 15 minutes be fore each activity assay. The temperature of the spectrophotometer was set to 25ºC and maintained constant throughout the assay.
- The time interval (5 minutes), lag time (10 seconds) and wavelength (340 nm) parameters were entered by using the menu in the kinetics mode of the spectrophotometer.
- The cuvettes were cleaned by distilled water before each use and the slides of the cuvettes were wiped off before each reading in the spectrophotometer. Finger prints or any other material on the exterior surface of cuvette and small bubbles or particulate substances on the interior solution affect the spectophotometric measurements and increase errors. Therefore, it is important to check and remove bubbles and be certain to wipe the cuvette after filling.
- The cuvette containing the blank solution (reaction mix without the sample) was placed to the cuvette holder to calibrate and auto zero the spectrophotometer. During calibration and the measurements of the samples it is important to make sure that the cuvette is aligned with the light.
- After calibration, PGM activity was determined for each sample by mixing 945µl of the reaction mix and 55µl of homogenate (for homogenaous mixing of the sample homogenate with the reaction mix a square of parafilm was placed over the mouth of the cuvette tube and inverted several times to thoroughly mix).
- Enzyme activity was measured by following the absorbance of NADH at 340 nm at 25ºC and initial rates were determined from change in OD every 10 seconds measured over the initial 5 minutes.
- PGM activity was expressed as units (micromoles of NADP reduced, per minute) per milligram of soluble protein.

2.2.3.3. Determination of Glycogen Content

To understand whether there is a correlation between PGM activity and glycogen amount, the samples that were used in the PGM activity measurements were assayed spectrophotometrically to determine the amount of glycogen in each sample.

Several dilutions of the sample homogenates were experimented to ensure that the glycogen concentration is within the range of the assay. In preliminary experiments the optimum dilution is determined to be 1:4. Dilutions of the samples were done with distilled water.

2.2.3.3.1. Procedure for Glycogen Measurements

- The assay protocol was performed by the optimization of the method used by Verrelli and Eanes (2001b) and carried out in flat-bottom 96-well microplate and spectrophotometric measurements were carried out on BioRad 680 microplate reader.
- Multichannel pipette was used for the pipetting of reaction mix to the samples to avoid volumetric errors and increase accuracy.
- Dilutions of glycogen standard and the samples were all run in duplicates. A schematic illustration of the loading of seven dilutions of glycogen standard and samples to 96-well microplate are given in Table 5.
- The reaction mix included one powder cap of glucose oxidase and peroxidase dissolved in 100 ml of distilled water with 1.6 ml of o-dianisidine dihydrochloride (50mg/ 20ml), 10 units of amyloglucosidase, and the mix was prepared immediately before performing the assay and placed on ice throughout assay.
- The assay contained 180µl of this mix and 20 µl of bee homogenate and was incubated in a 37ºC water bath for 30 minutes before immediate transfer to ice.

- The measurements are performed at OD_{450} and glycogen concentrations were determined from glycogen standards.
- Concentrations were expressed as milligrams of glycogen per milligram of soluble protein.

The blank, glycogen standards, and the samples are loaded to the plates in replicates in a pattern as shown in the Table 2.4.

Table 2.4. Pattern of sample loading to 96-well microplates for glycogen measurements.

	1	**2**	**3→→→12**
A	**blank**	**blank**	**SAMPLES IN DUBLICATES**
B	Glycogen Standard 0.2 mg/ml	Glycogen Standard 0.2 mg/ml	
C	Glycogen Standard 0.4 mg/ml	Glycogen Standard 0.4 mg/ml	
D	Glycogen Standard 0.8 mg/ml	Glycogen Standard 0.8 mg/ml	
E	Glycogen Standard 1.0 mg/ml	Glycogen Standard 1.0 mg/ml	
F	Glycogen Standard 1.2 mg/ml	Glycogen Standard 1.2 mg/ml	
G	Glycogen Standard 1.4 mg/ml	Glycogen Standard 1.4 mg/ml	
H	Glycogen Standard 2.0 mg/ml	Glycogen Standard 2.0 mg/ml	

2.2.3.4. Soluble Protein Measurements by Bradford Assay

In this study protein concentration was measured to compare and correlate PGM enzyme activity and glycogen content of different *Pgm* genotypes, by determination of the protein content of the homogenate fractions used in all assays, enzyme activity and glycogen measurements are standardized by the amount of soluble protein available in the homogenates. Therefore it is important to make accurate measurements of protein concentration of the samples used in the analysis of enzyme activity and glycogen content since calculations of activity and glycogen amount is can be further magnified by any error that occur in the data obtained from the protein measurements.

The selection of the method employed in a study is an important step in a protein assay. The composition of the protein sample and the buffer used in the assay, or in preparation of the homogenates are important factors that should be considered in the choice of the method (Bio-Rad Protein Assay Instruction Manual, 2007).

In this study the BioRad Bradford protein assay was used for the determination of total protein in homogenates. The BioRad Bradford protein assay is a dye-binding assay that employs the use of Coomassie G-250 Dye in a colorimetric reagent for the detection and quantitation of total protein and the method was first described by Dr. Marion Bradford in 1976 (Bradford, 1976; Bio-Rad Protein Assay Instruction Manual, 2007). The Coomassie blue dye binds to positively charged residues (lysine, arginine, and histidine) and aromatic residues of the protein, especially arginine, which in turn leads to the development of a differential color change of dye in response to various concentrations of protein (Bradley and Markwell, 2007).

The formation of the dye-protein complex results in a color change from the reddish/brown form of the dye (absorbance maximum at 465 nm) to the blue form of the dye (absorbance maximum at 610 nm) protein and the spectrophotometric reading of the blue color can be done at any wavelength between 575 nm and 615 nm, at the two extremes (575 nm and 615 nm) (Bradley and Markwell, 2007). An advantage of the Bradford assay is that it does not require critical timing since the absorbance of the dye-protein complex is more stable as compared to other methods, and also it is quite sensitive; it can detect from 0.2 to 1.4 mg of protein per ml (Bio-Rad Protein Assay Instruction Manual, 2007).

2.2.3.4.1. Procedure of the assay

Several dilutions of the sample homogenates were experimented to ensure that the protein concentration is within the range of the assay. In preliminary experiments the optimum dilution was determined to be 1:4. Dilutions of the samples were done with distilled water. Measurements were conducted by using a protein assay from BioRad

(kit number and code given in Appendix A) that employs Bradford Method. The procedure used in the assays was optimized from Bio-Rad Protein Assay Instruction Manual (2007) and Verrelli and Eanes (2001b).

The assay protocol was carried out in flat-bottom 96-well microplates. Multichannel pipette is used for the pipetting of dye reagent to the samples to avoid volumetric errors and increase accuracy. Dilutions of standard protein (BSA) and the samples are all run in duplicates. A schematic illustration of the loading of seven dilutions of BSA standard and samples to 96-well microplate is given in Table 2.5.

- The dye reagent was preapared by diluting 1 volume Dye Reagent Concentrate with 4 volume distilled, deionized (DDI) water. It is then filtered through Whatman #1 filter to remove particulates. This diluted reagent may be used for approximately 2 weeks when kept at room temperature.
- It is advised to prepare three to five dilutions of a protein standard, which is representative of the protein solution to be tested. The linear range of the assay for BSA is 0.2 to 0.9 mg/ml, whereas in this study seven dilutions of BSA were prepared in a range of 0.2-1.4 to increase sensitivity and accuracy.
- 5 µl of each standard and sample homogenate were loaded into separate wells carefully.
- 250 µl of diluted dye reagent were added to each well.
- The plates were incubated at room temperature for 10 minutes.
- To determine the protein concentration in each well measurements were performed at 570 nm in BioRad plate reader.
- OD_{570} versus concentrations of the standards were plotted and protein concentrations of the samples were determined from the standard curve.

Table 2.5. Pattern of sample loading to 96-well microplates for glycogen measurements.

	1		**2**		**3→→→→→→→12**
A	**blank**		**blank**		
B	BSA Standard	0.2 mg/ml	BSA Standard	0.2 mg/ml	
C	BSA Standard	0.4 mg/ml	BSA Standard	0.4 mg/ml	
D	BSA Standard	0.6 mg/ml	BSA Standard	0.6 mg/ml	**SAMPLES IN**
E	BSA Standard	0.8 mg/ml	BSA Standard	0.8 mg/ml	**DUBLICATES**
F	BSA Standard	1.0 mg/ml	BSA Standard	1.0 mg/ml	
G	BSA Standard	1.2 mg/ml	BSA Standard	1.2 mg/ml	
H	BSA Standard	1.4 mg/ml	BSA Standard	1.4 mg/ml	

2.2.4. Statistical Analysis

2.2.4.1. Analysis of Seasonal Allozyme Variation

Microevolution has been defined as a change in allele frequencies in populations through time (Mayr, 1963). Allele frequencies are the basic genetic parameters of a population and can be calculated simply from the counts of alleles in allozyme genotypes. Allele frequencies were calculated as the proportion of the observed allele to the total number of alleles in the population, and this can be formulated as:

$$\text{Freq. of an Allele (p)} = \frac{(2x\ N_{\text{Homozygotes}}) + (N_{\text{Heterozygotes}})}{2x\ \text{Total Number of Individuals (N)}} \qquad (2.4)$$

Genotype frequencies are other basic population genetic parameters that are commonly used in explaining the structure of a population. In allozyme analysis, the

numbers of homozygote and heterozygote individuals are determined by the examination of the allozyme patterns on the gel and observed genotype frequencies are computed as the proportion of the observed genotype to the total number of individuals in the sample.

Observed and expected heterozygosities are calculated in order to determine the genetic structure of a population. Observed heterozigosity is calculated with the formula below:

$$\text{Observed Heterozigosity} = \frac{\text{Number of Heterozygotes}}{\text{Total Number of Individuals}} \qquad (2.5)$$

In this study, number of alleles, number of genotypes, allele and genotype frequencies at *Pgm* and *Hk* loci were calculated both for each month of sample collection and the total samples for all populations. Expected heterozygosity is computed from the allelic frequencies calculated from the population data meeting Hardy-Weinberg expectations. Heterozygosities with Leven's correction which is the same as Nei's unbiased heterozygosity have been calculated by using POPGENE on the web software (Yeh and Yang, 1999)

Goodness-of-fit (G^2) tests were used for the computation of the expected genotypic frequencies and for the determination of whether the observed frequencies are consistent with Hardy-Weinberg predictions. Both of these calculations have been performed by using POPGENE on the web software (Yeh and Yang, 1999)

2.2.4.2. Analysis of Protein and Glycogen Concentration

For the determination of the protein and glycogen concentrations in the samples, the absorbance readings of the standards were used for the construction of the standard curves. Standards used in protein and glycogen assays represent the set of dilutions in which the BSA or glycogen was present in regularly increasing, known concentrations. The absorbance data obtained from readings of the standards at their particular wavelengths (570nm for protein measurements and 450nm for glycogen measurements) was used to construct the standard curve which was consequently used to convert the absorbance readings of the samples into their corresponding protein and glycogen concentrations.

2.2.4.2.1. Graphing the standard curve

The data obtained from Bradford and glycogen assays were recorded on a spreadsheet in Excel so that the first column contained the protein and glycogen concentrations of the standards and the second column was the corresponding absorbance at 570 nm and 450nm respectively. X-y scatter graphs with the protein concentration on the y axis, and the absorbance on the x axis were obtained. R^2 values for the curves and the linear regression equation that uses the "least squares" method to give a straight line that best fits the absorbance data were obtained for each block of samples. The Y-intercept and the slope of the lines were used for the determination of the glycogen and protein concentrations in the samples. Protein and glycogen concentrations of the samples were calculated according to the following formulation:

$$[(\text{Sample OD-Y intercept}) / (\text{slope of the line})] \quad (\text{Dilution Factor}) \qquad (2.6)$$

The program gives the equation of a straight line as y = mx + b. Thus the equation for the concentration of the unknown sample becomes:

$$[\text{sample glycogen or protein}] = (OD_{sample}\text{-b})(1/m)(\text{dilution factor}). \qquad (2.7)$$

Dilution Factor = the dilution factor is the final volume of the assay divided by the volume of fraction of homogenate.

A standard curve for the BioRad Protein Assay is given in Figure 2.5. Protein and glycogen concentrations of each block of 10 samples were determined from their corresponding standard curves. The mean and standard deviation values were determined for each group.

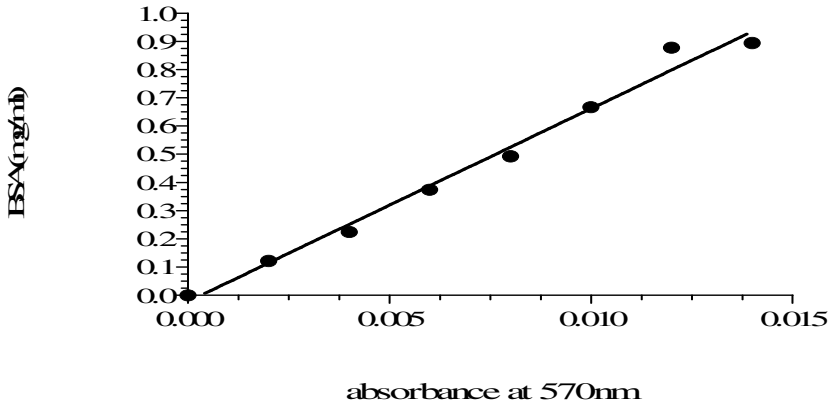

Figure 2.5. Standard curve for the BioRad Protein Assay (slope=0.14432, R^2=0.9869, y=0.14432x+0.000407)

2.2.4.3. Analysis of Enzyme Kinetic Data

Enzymatic activity was determined by measuring the increase in absorbance at 340 nm at 25°C, pH 7.4, caused by the reduction of NADP which is illustrated in the following reactions:

PGM

Glucose-1-P ⟶ **Glucose-6-P** (2.8)

↓ G-1,6-diphosphate

Glucose-6-P +NADP ⟶ **Gluconate-6-phosphate+NADPH**

Mg^{+2}

This enzyme assay is based on the measurement of the conversion of $NADP^+$ to NADPH, therefore it is not a direct measure of PGM activity; it measures the rate of a secondary reaction, in a glucose-6-phosphate dehydrogenase coupled system (Najjar and Pullman, 1955).

In this study, PGM activity was calculated for each block of samples by determination of the slope (ΔA/min) from the linear regression equation that uses the "least squares" method to give a straight line that best fits OD_{340} vs. time data. Beer's law was used to convert ΔA_{340}/min values to µmol/min, in other words, activity readings of absorbance units were used for the determination of units of concentration.

Several factors of the assay conditions were combined with the Beer's law to convert absorbance readings into the changes in NADH concentration:

- Total volume of assay
- Volume of homogenate fraction used in the assay
- Dilution factor for each fraction analyzed

Thus the equation for Pgm activity becomes:

µmol of NADP reduced/min/ml = (ΔA-340 nm/min) X (1ml assay volume/6.2) X (1/0.055ml extract) X (dilution factor)

(2.9)

All extracts were assayed in replicates, and the averages and standard deviations activities were calculated by Sigma Plot-GraphPad. Means were compared using unpaired, two-tailed *t*-tests and *P*-values were given in the results (Sokal and Rohlf, 1995).

CHAPTER 3

RESULTS

3.1. Allozyme Data

3.1.1.Polymorphism at Pgm and Hk Loci in Kırklareli

(A. mellifera carnica) Population

A total number of 1300 (26 month ×50 individuals/month) worker bees were studied in the electrophoretic analysis of *Pgm* and *Hk* loci of samples collected from Kırklareli. The genotypes of all allozymes are designated by using the relative mobilities with the fastest allozyme used as the standard (relative mobility=1.00).

PGM is a monomeric enzyme with one locus in honeybee genome. Four different alleles were observed in the electrophoretic analysis of Kırklareli samples; they were called as *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 mobility (Figure 3.1. and 3.2.). Among the observed alleles, *Pgm-65* and *Pgm-110* were the rare alleles having the smallest frequencies both in the total number of bees analyzed (Table 3.1) and in each of the 26 months from October 2005 to December 2007 (Table 3.3). Alleles *Pgm-75 and Pgm-100* comprised the 99.2% of all alleles observed.

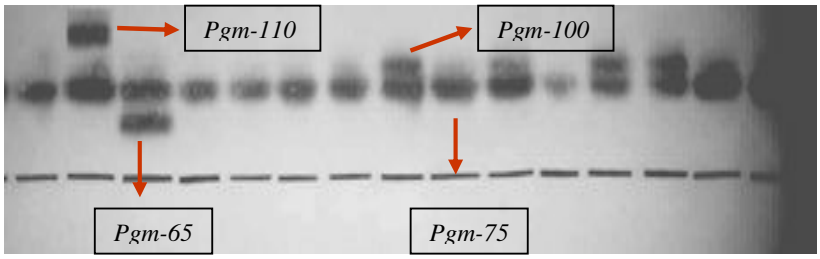

Figure 3.1. Alleles observed in the electrophoretic analysis of *Pgm* locus.

Figure 3.2. Alleles observed in the electrophoretic analysis of *Hk* locus.

Table 3.1. Distribution of the observed *Pgm* alleles in samples collected from Kırklareli

Allele	**Count**	**Percentage**
Pgm-65	16	0.61
Pgm-75	1774	68.2
Pgm-100	805	31.0
Pgm-110	5	0.19
TOTAL	2600	100.0

Table 3.2. Distribution of the observed *Pgm* genotypes in samples collected from Kırklareli.

Genotype	Count	Percentage
Pgm-75/100	797	61.30
Pgm-75/75	478	36.76
Pgm-100/100	3	0.23
Pgm-65/75	16	1.23
Pgm-65/100	1	0.07
Pgm 75/110	5	0.38
TOTAL	1300	100

The homozygous genotype for the rare alleles (*Pgm-65 and Pgm-110*) has not been observed among the 1300 individuals studied (Table 3.2. and Table 3.5.). Furthermore the frequencies of their heterozygote genotypes (*Pgm-65/100, Pgm-75/110, and Pgm-65/75*) were quite small (Table 3.5).

Like PGM, HK is a monomeric enzyme and at *Hk* locus two different alleles were observed throughout the electrophoretic analysis of Kırklareli samples: *Hk-87* and *Hk-100* (frequencies of alleles given in Table 3.3.). *Hk-100* was the most common allele in this locus. In electromorps, two different genotypes were observed: *Hk-87/100* and *Hk- 100/100.* The genotype frequencies at *Hk* locus for each month together with the *P* values of likelihood ratio tests are given in Table 3.6.

The heterozygosity values of Kırklareli samples for each month are given in Table 3.7. For the samples collected in winter the mean heterozygosity at *Pgm* locus is 0.935 and for the samples collected in summer months the average heterozygosity is 0.143. The frequency of the *Pgm-75/100* heterozygotes differed significantly between summer and winter months ($P < 0.001$).

Accordingly, the frequencies of the *Pgm* alleles (*Pgm-75 and Pgm-100*) differed significantly between winter and summer samples ($P < 0.001$). The genotype

frequencies of the samples collected in summer were found to be in Hardy-Weinberg equilibrium in contrast to the winter samples which showed significant levels of deviations. The comparisons of allele frequencies of winter and summer samples are tabulated in Table 3.3 for each month. The deviations of the genotype frequencies from the equilibrium were tested by likelihood ratio test (G^2) and the results are given in Table 3.4

Table 3.3. Allele frequencies at *Pgm* (*Pgm-65, Pgm-75, Pgm-100, Pgm-110*) and *Hk* (*Hk-87, Hk-100*) loci for each month from October 2005 to December 2007 for Kırklareli (*A. mellifera carnica*) population.

Sample Collection month and year (n=50 / month)	**ALLELE FREQUENCIES**					
	Pgm-75	***Pgm-100***	***Pgm-65***	***Pgm-110***	***Hk-100***	***Hk-87***
October '05	0.64	0.35	0.01	0.00	0.95	0.05
November '05	0.59	0.39	0.00	0.02	0.94	0.06
December '05	0.56	0.42	0.02	0.00	0.93	0.07
January '06	0.52	0.48	0.00	0.00	0.94	0.06
February '06	0.52	0.48	0.00	0.00	0.95	0.05
March '06	0.51	0.49	0.00	0.00	0.92	0.08
April '06	0.67	0.32	0.01	0.00	0.94	0.06
May '06	0.83	0.16	0.00	0.00	0.95	0.05
June '06	0.91	0.08	0.00	0.01	0.95	0.05
July '06	0.93	0.07	0.00	0.00	0.92	0.08
August '06	0.95	0.05	0.00	0.00	0.94	0.06
September '06	0.61	0.36	0.02	0.01	0.92	0.08
November '06	0.61	0.37	0.01	0.01	0.93	0.07
December '06	0.55	0.45	0.00	0.00	0.96	0.04
January '07	0.53	0.46	0.01	0.00	0.95	0.05
February '07	0.53	0.47	0.00	0.00	0.94	0.06
March '07	0.52	0.48	0.00	0.00	0.95	0.05
April '07	0.70	0.28	0.02	0.00	0.96	0.04
May '07	0.81	0.18	0.01	0.00	0.93	0.07
Jun '07	0.89	0.10	0.01	0.00	0.96	0.04
July '07	0.91	0.09	0.00	0.00	0.95	0.05
August '07	0.96	0.04	0.00	0.00	0.92	0.08
September '07	0.66	0.32	0.02	0.00	0.96	0.04
October '07	0.63	0.36	0.01	0.00	0.94	0.06
November '07	0.64	0.35	0.01	0.00	0.93	0.07
December '07	0.56	0.44	0.00	0.00	0.95	0.05

Table 3.4. Frequencies of *Pgm-75/75* and *Pgm-75/100*, likelihood ratio test values (G^2) and corresponding probabilities for each month from October 2005 to December 2007 for Kırklareli (*A. mellifera carnica*) population.

Sample collection month and year (n=50 / month)	**GENOTYPE FREQUENCIES**			
	Pgm- 75/100	***Pgm- 75/75***	***G^2***	***P***
October '05	0.70	0.28	20.91	0.000
November '05	0.78	0.18	30.69	0.000
December '05	0.84	0.12	38.70	0.000
January '06	0.92	0.06	41.47	0.000
February '06	0.96	0.04	54.20	0.000
March '06	0.98	0.02	59.89	0.000
April '06	0.60	0.36	9.34	0.025
May '06	0.32	0.66	3.30	0.347
June '06	0.16	0.82	0.79	0.851
July '06	0.14	0.86	0.45	0.501
August '06	0.10	0.90	0.21	0.646
September '06	0.70	0.24	22.74	0.000
November '06	0.74	0.22	26.35	0.000
December '06	0.86	0.12	31.03	0.000
January '07	0.92	0.06	49.52	0.000
February '07	0.94	0.06	49.52	0.000
March '07	0.96	0.04	54.20	0.000
April '07	0.56	0.40	12.85	0.004
May '07	0.36	0.62	4.26	0.234
Jun '07	0.2	0.78	1.23	0.743
July '07	0.18	0.82	0.79	0.373
August '07	0.08	0.92	0.12	0.723
September '07	0.64	0.32	12.81	0.005
October '07	0.72	0.26	22.60	0.000
November '07	0.70	0.28	15.31	0.001
December '07	0.88	0.12	38.70	0.000

Table 3.5. Frequencies of rare *Pgm* genotypes for each month from October 2005 to December 2007 for Kırklareli (*A. mellifera carnica*) population.

Sample collection month and year (n=50 / month)	**FREQUENCIES OF RARE GENOTYPES**			
	Pgm-100/100	***Pgm-65/75***	***Pgm-65/100***	***Pgm-75/110***
October '05	0.00	0.02	0.00	0.00
November '05	0.00	0.00	0.00	0.04
December '05	0.00	0.04	0.00	0.00
January '06	0.02	0.00	0.00	0.00
February '06	0.00	0.00	0.00	0.00
March '06	0.00	0.00	0.00	0.00
April '06	0.02	0.02	0.00	0.00
May '06	0.00	0.02	0.00	0.00
June '06	0.00	0.00	0.00	0.02
July '06	0.00	0.00	0.00	0.00
August '06	0.00	0.00	0.00	0.00
September '06	0.00	0.02	0.02	0.02
November '06	0.00	0.02	0.00	0.02
December '06	0.02	0.00	0.00	0.00
January '07	0.00	0.02	0.00	0.00
February '07	0.00	0.00	0.00	0.00
March '07	0.00	0.00	0.00	0.00
April '07	0.00	0.04	0.00	0.00
May '07	0.00	0.02	0.00	0.00
Jun '07	0.00	0.02	0.00	0.00
July '07	0.00	0.00	0.00	0.00
August '07	0.00	0.00	0.00	0.00
September '07	0.00	0.04	0.00	0.00
October '07	0.00	0.02	0.00	0.00
November '07	0.00	0.02	0.00	0.00
December '07	0.00	0.00	0.00	0.00

Table 3.6. Genotype frequencies at *Hk* locus and corresponding probabilities of likelihood ratio tests for each month from October 2005 to December 2007 for Kırklareli (*A. mellifera carnica*) population.

Sample collection month and year (n=50 / month)	**GENOTYPE FREQUENCIES**		
	Hk-100/100	***Hk-87/100***	***P*-values**
October '05	0.90	0.10	0.6462
November '05	0.88	0.12	0.5719
December '05	0.86	0.14	0.5013
January '06	0.88	0.12	0.5719
February '06	0.90	0.10	0.6462
March '06	0.84	0.16	0.5669
April '06	0.88	0.12	0.5719
May '06	0.88	0.12	0.5719
June '06	0.90	0.10	0.6462
July '06	0.84	0.16	0.5669
August '06	0.88	0.12	0.5719
September '06	0.84	0.16	0.5669
November '06	0.86	0.14	0.5013
December '06	0.92	0.08	0.7995
January '07	0.90	0.10	0.6462
February '07	0.88	0.12	0.5719
March '07	0.90	0.10	0.6462
April '07	0.92	0.08	0.7995
May '07	0.86	0.14	0.5013
Jun '07	0.92	0.08	0.7995
July '07	0.90	0.10	0.6462
August '07	0.84	0.16	0.5669
September '07	0.92	0.08	0.7995
October '07	0.88	0.12	0.5719
November '07	0.86	0.14	0.5013
December '07	0.90	0.10	0.6462

In contrast to the *Pgm* locus, the allele and genotype frequencies of the *Hk* did not differ temporarily (Table 3.3. and Table 3.6.). The genotype frequencies are found to be in Hardy-Weinberg equilibrium in both summer and winter months independent of the sample collection time (Table 3.6).

Table 3.7. Observed and expected heterozygosity values for *Pgm* and *Hk* loci.

Sample Collection month and year (n=50 /month)	**Observed and Expected Heterozygosity Values**			
	Observed *-Pgm-*	**Expected *-Pgm-***	**Observed *-Hk-***	**Expected *-Hk-***
October '05	0.7200	0.4725	0.1000	0.0960
November '05	0.8200	0.5044	0.1200	0.1139
December '05	0.8800	0.5147	0.1400	0.1315
January '06	0.9200	0.5042	0.1200	0.1139
February '06	0.9600	0.5042	0.1000	0.0960
March '06	0.9800	0.5048	0.0800	0.0776
April '06	0.6200	0.4531	0.1200	0.1139
May '06	0.3400	0.2883	0.1000	0.0960
June '06	0.1800	0.1671	0.1000	0.0960
July '06	0.1400	0.1315	0.0800	0.0776
August '06	0.1000	0.0950	0.1200	0.1139
September '06	0.7600	0.5028	0.0800	0.0776
November '06	0.7800	0.4958	0.1400	0.1315
December '06	0.8657	0.5469	0.1178	0.1096
January '07	0.9400	0.5125	0.1000	0.0960
February '07	0.9400	0.5032	0.1200	0.1139
March '07	0.9600	0.5042	0.1000	0.0960
April '07	0.6000	0.4356	0.1178	0.1096
May '07	0.3800	0.3145	0.1400	0.1315
Jun '07	0.1800	0.1671	0.1345	0.1278
July '07	0.1800	0.1655	0.1000	0.0960
August '07	0.0800	0.0776	0.0800	0.0776
September '07	0.6800	0.4663	0.1345	0.1278
October '07	0.7400	0.4782	0.1200	0.1139
November '07	0.7200	0.4725	0.1400	0.1315
December'07	0.8800	0.4978	0.1000	0.0960

In contrast to the *Pgm* locus, the heterozygosity values remained stable throughout the year at *Hk* locus. The mean heterozygosity of the all samples studied was found to be 0.112, mean heterozygosity values for summer and winter samples were computed to be 0.104 and 0.110 respectively.

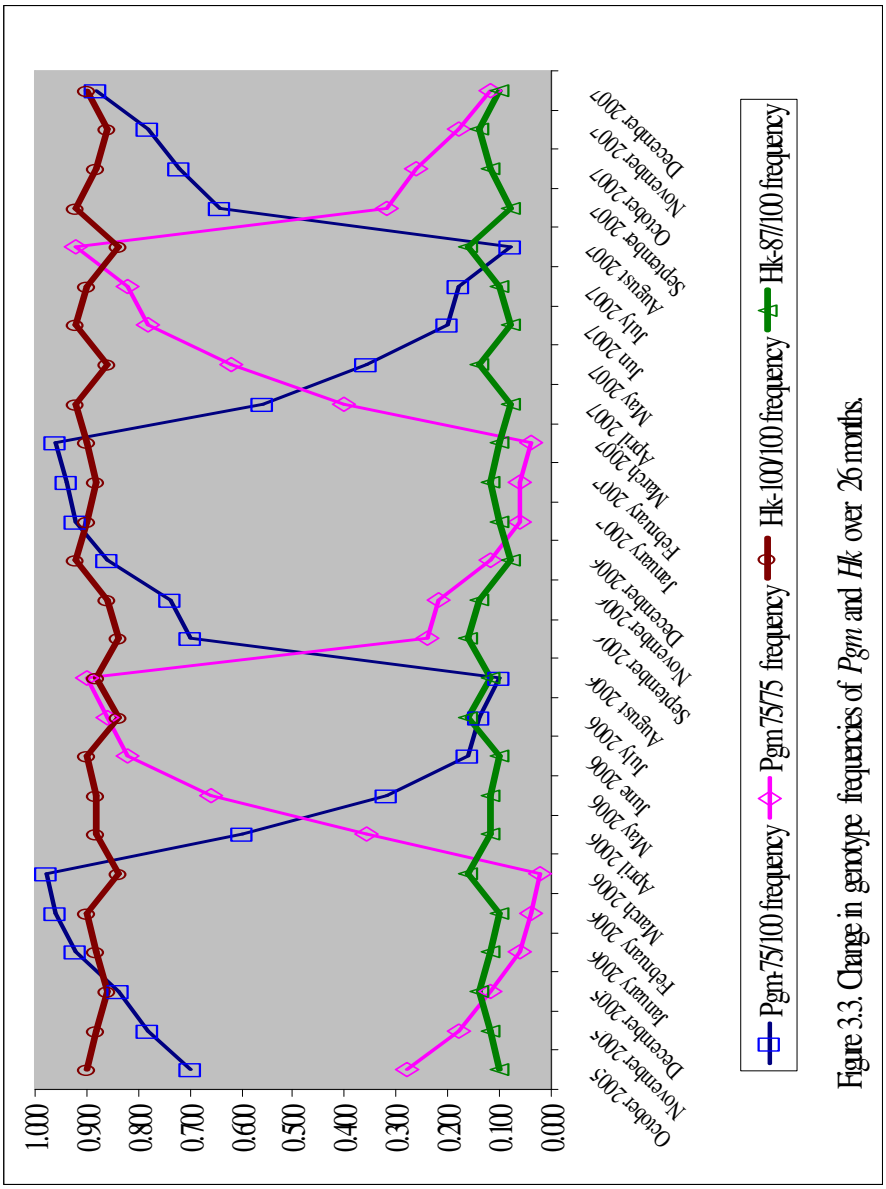

Figure 3.3. Change in genotype frequencies of *Pgm* and *Hk* over 26 months.

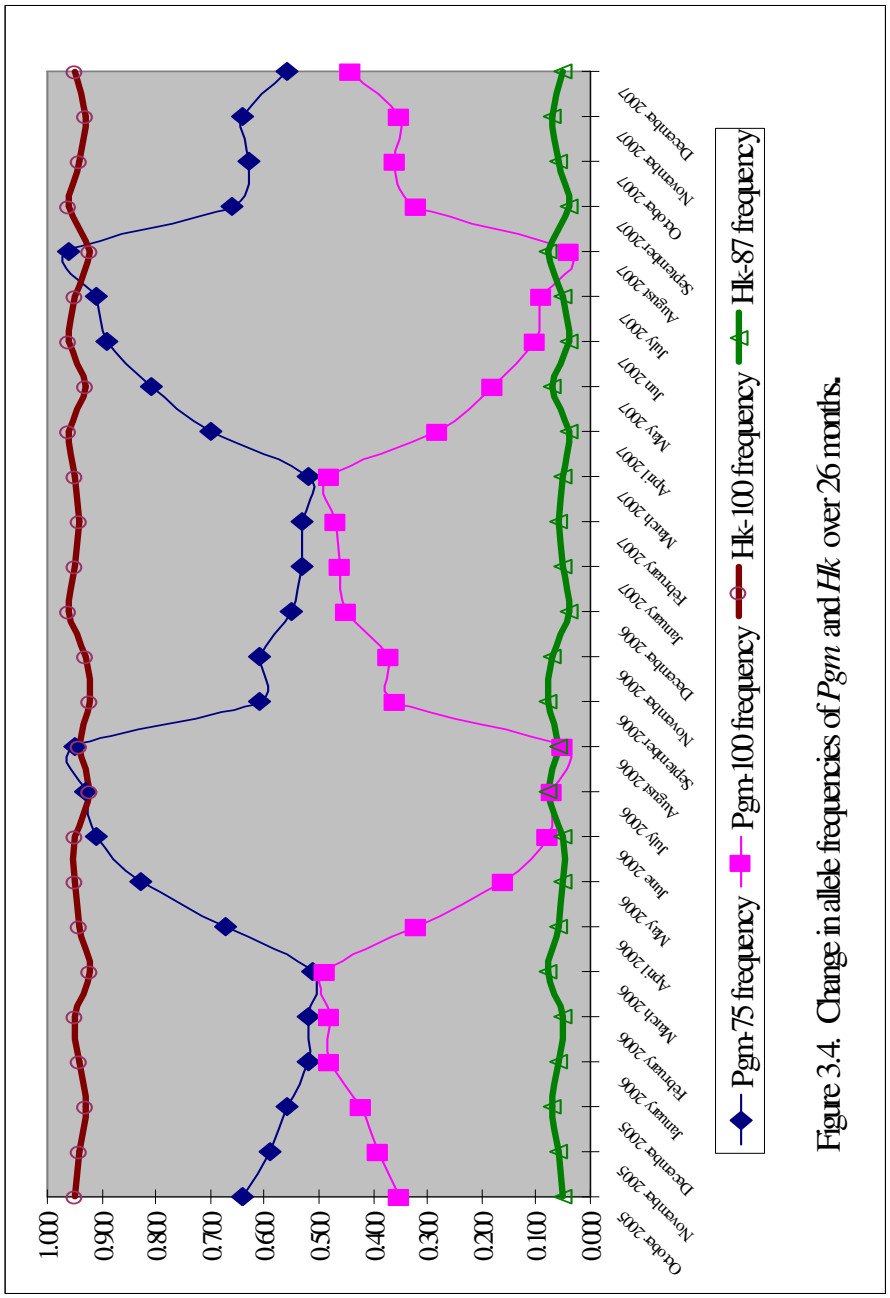

Figure 3.4. Change in allele frequencies of *Pgm* and *Hk* over 26 months.

3.1.2. Polymorphism at *Pgm* and *Hk* Loci in Artvin (*A.mellifera caucasica*) Population

A total number of 680 (17 month ×40 individuals/month) worker bees were studied in the electrophoretic analysis of *Pgm* and *Hk* loci of samples collected from Artvin.

Among the four alleles observed (*Pgm-65, Pgm-75, Pgm-100, Pgm-110*), *Pgm-65* and *Pgm-110* were the rare alleles with the smallest frequencies both in the total number of bees analyzed (Table 3.8) and in each of the 17 months from July 2006 to December 2007 (Table 3.10). Alleles *Pgm-75* and *Pgm-100* comprised the 99.27 % of all alleles observed with *Pgm-75* being the most common allele in the Artvin population.

Table 3.8. Distribution of the observed *Pgm* alleles in samples collected from Artvin.

Allele	**Count**	**Percentage**
Pgm-65	7	0.51
Pgm-75	860	68.76
Pgm-100	415	30.51
Pgm-110	3	0.22
TOTAL	1360	100.0

As in the case of the Kırklareli samples, the homozygous genotype for the rare alleles (*Pgm-65 and Pgm-110*) has not been observed among the 680 individuals studied. In addition, *Pgm-100/100* and *Pgm-65/100* which were detected in very low frequencies in Kırklareli samples were not observed in Artvin population (Table 3.12).

Table 3.9. Distribution of the observed *Pgm* genotypes in samples collected from Artvin.

Genotype	Count	Percentage
Pgm-75/100	415	61.04
Pgm-75/75	255	37.5
Pgm-100/100	0	0
Pgm-65/75	7	1.02
Pgm-65/100	0	0
Pgm 75/110	3	0.44
TOTAL	680	100

Alleles observed at *Hk* locus in Artvin region were the same as in the analysis of Kırklareli samples: *Hk-87* and *Hk-100* (frequencies of alleles given in Table 3.10). *Hk-100* was the most common allele in this locus. The genotypes observed were: *Hk-87/100* and *Hk-100/100* and their frequencies for each month are given in Table 3.13.

The observed heterozygosity values of Artvin samples computed for each month are given in Table 3.14. At *Pgm* locus, samples collected in winter months had mean heterozygosity of 0.935 and for the samples collected in summer months the average heterozygosity is 0.143. The frequency of the *Pgm-75/100* heterozygotes differed significantly between summer and winter months ($P< 0.001$).

Accordingly, the frequencies of the *Pgm* alleles (*Pgm-75* and *Pgm-100*) differed significantly between winter and summer samples ($P< 0.001$). The allele frequencies of the samples collected in summer were found to be in Hardy-Weinberg equilibrium in contrast to the winter samples which showed significant levels of deviations. The comparisons of allele frequencies of winter and summer samples are tabulated in Table 3.10 for each month. The deviations of the genotype frequencies from the equilibrium were tested by likelihood ratio test (G^2) and the results are given in Table 3.11.

Table 3.10. Allele frequencies at *Pgm* and *Hk* loci for each month from July 2006 to December 2007 for Artvin (*A. mellifera caucasica*) population.

Sample Collection month and year (n=40 / Month)	ALLELE FREQUENCIES					
	Pgm -75-	***Pgm -100-***	***Pgm -65-***	***Pgm -110-***	***Hk -100-***	***Hk -87-***
July 2006	0.96	0.04	0.00	0.00	0.925	0.075
August2006	0.98	0.02	0.00	0.00	0.9	0.1
September 2006	0.70	0.29	0.01	0.00	0.925	0.075
November 2006	0.59	0.40	0.01	0.00	0.95	0.05
December 2006	0.56	0.42	0.00	0.02	0.9	0.1
January 2007	0.46	0.54	0.00	0.00	0.925	0.075
February 2007	0.50	0.50	0.00	0.00	0.95	0.05
March 2007	0.52	0.48	0.00	0.00	0.9	0.1
April 2007	0.59	0.40	0.01	0.00	0.925	0.075
May 2007	0.70	0.29	0.00	0.01	0.95	0.05
Jun 2007	0.80	0.18	0.01	0.01	0.9	0.1
July 2007	0.94	0.06	0.00	0.00	0.95	0.05
August 2007	0.96	0.04	0.00	0.00	0.95	0.05
September 2007	0.70	0.29	0.01	0.00	0.925	0.075
October 2007	0.63	0.35	0.02	0.00	0.925	0.075
November 2007	0.57	0.42	0.00	0.01	0.90	0.1
December 2007	0.54	0.46	0.00	0.00	0.95	0.05

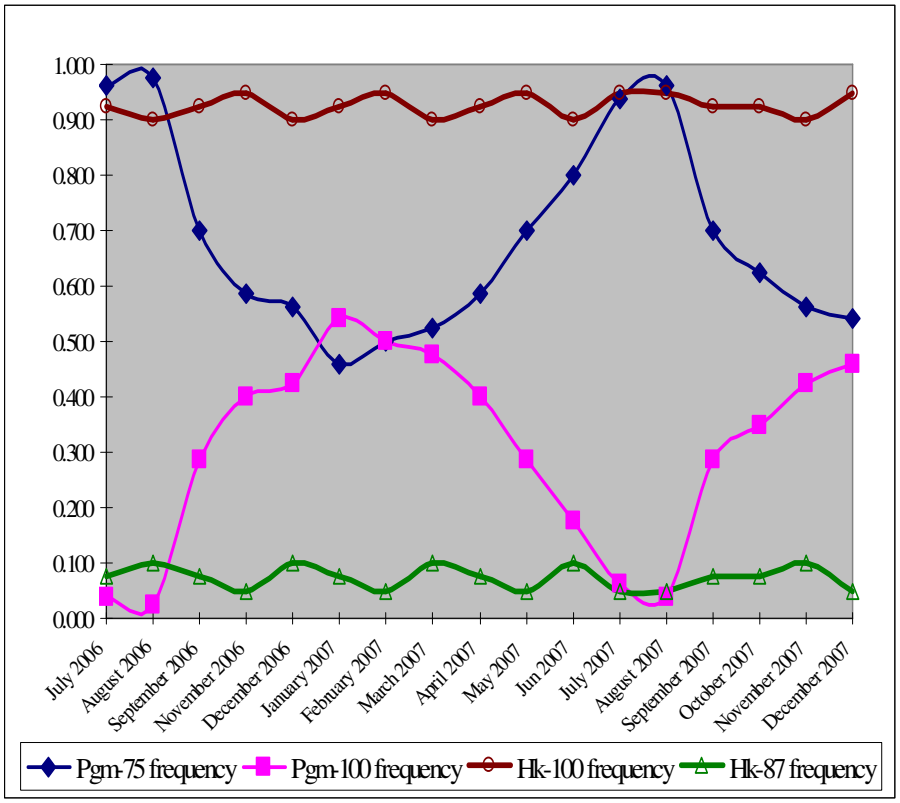

Figure 3.5. Change in allele frequencies of *Pgm* and *Hk* loci over 17 months in Artvin (*A. mellifera caucasica*) samples.

Table 3.11. Frequencies of *Pgm 75/75* and *Pgm 75/100* for each month for Artvin (*A. mellifera caucasica*) population.

Sample collection month and year (n=40 / Month)	**GENOTYPE FREQUENCIES**			
	Pgm-75/100	***Pgm-75/75***	G^2	***P***
July '06	0.075	0.925	0.077	0.780
August '06	0.05	0.95	0.025	0.872
September '06	0.625	0.375	12.57	0.005
November '06	0.8	0.175	24.88	0.000
December '06	0.85	0.125	30.20	0.000
January '07	0.9	0.1	25.97	0.000
February '07	1.0	0.00	31.84	0.000
March '07	0.95	0.05	39.00	0.000
April '07	0.8	0.175	15.36	0.001
May '07	0.575	0.4	10.19	0.016
Jun '07	0.35	0.6	12.57	0.050
July '07	0.125	0.875	0.266	0.605
August '07	0.075	0.925	0.077	0.780
September '07	0.575	0.4	10.19	0.016
October '07	0.7	0.25	18.67	0.000
November '07	0.85	0.125	30.20	0.000
December '07	0.925	0.075	36.98	0.000

Table 3.12. Frequencies rare genotypes at *Pgm* locus for each month from July 2006 to December 2007 for Artvin (*A. mellifera caucasica*) population.

Sample collection month and year (n=40 / Month)	**GENOTYPE FREQUENCIES**			
	Pgm-100/100	***Pgm-65/75***	***Pgm-65/100***	***Pgm-75/110***
July '06	0.00	0.00	0.00	0.00
August '06	0.00	0.00	0.00	0.00
September '06	0.00	0.025	0.00	0.00
November '06	0.00	0.025	0.00	0.00
December '06	0.00	0.00	0.00	0.025
January '07	0.00	0.00	0.00	0.00
February '07	0.00	0.00	0.00	0.00
March '07	0.00	0.00	0.00	0.00
April '07	0.00	0.025	0.00	0.00
May '07	0.00	0.00	0.00	0.025
Jun '07	0.00	0.025	0.00	0.025
July '07	0.00	0.00	0.00	0.00
August '07	0.00	0.00	0.00	0.00
September '07	0.00	0.025	0.00	0.00
October '07	0.00	0.05	0.00	0.00
November '07	0.00	0.00	0.00	0.025
December '07	0.00	0.00	0.00	0.025

Table 3.13. Genotype frequencies at *Hk* locus over 17 months for Artvin samples.

Sample collection month and year (n=40 / Month)	**GENOTYPE FREQUENCIES**		
	Hk-100/100	***Hk-87/100***	***P***
July '06	0.85	0.15	0.780
August '06	0.8	0.2	0.670
September '06	0.85	0.15	0.780
November '06	0.9	0.1	0.869
December '06	0.8	0.2	0.670
January '07	0.85	0.15	0.780
February '07	0.9	0.1	0.869
March '07	0.8	0.2	0.670
April '07	0.85	0.15	0.780
May '07	0.9	0.1	0.869
Jun '07	0.8	0.2	0.670
July '07	0.9	0.1	0.869
August '07	0.9	0.1	0.869
September '07	0.85	0.15	0.780
October '07	0.85	0.15	0.780
November '07	0.8	0.2	0.670
December '07	0.9	0.1	0.869

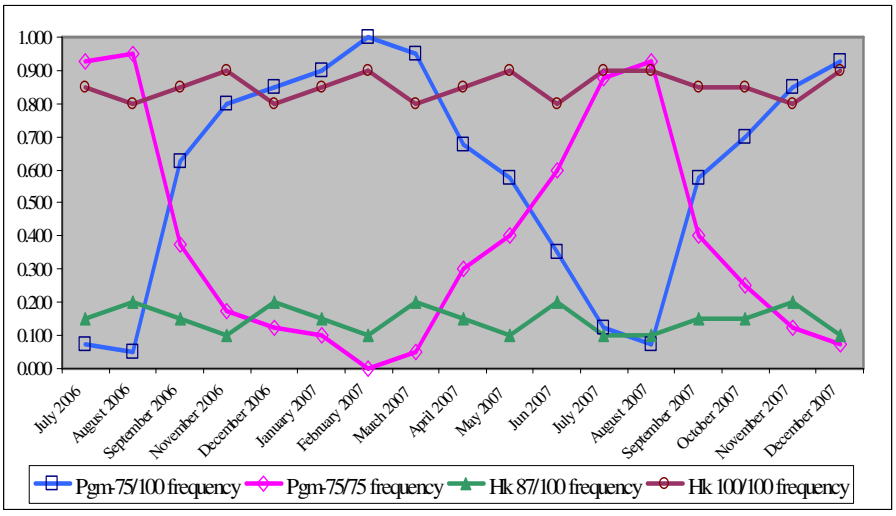

Figure 3.6. Change in genotype frequencies of *Pgm* and *Hk* loci over 17 months in Artvin (*A. mellifera caucasica*) samples

Table 3.14. Observed and expected heterozygosity values for *Pgm* and *Hk* loci for each month from July 2006 to December 2007 for Artvin (*A. mellifera caucasica*) population.

Sample Collection month and year (n=40 / month)	**Observed and Expected Heterozygosity Values**			
	Observed *Pgm*	**Expected *Pgm***	**Observed *Hk***	**Expected *Hk***
July '06	0.0750	0.0731	0.0896	0.0783
August '06	0.0500	0.0494	0.0789	0.0659
September '06	0.6500	0.4522	0.0896	0.0783
November '06	0.7956	0.5275	0.1078	0.0965
December '06	0.8250	0.5009	0.0789	0.0659
January '07	0.8750	0.5092	0.0859	0.0732
February '07	0.9000	0.5013	0.0789	0.0659
March '07	0.9500	0.5051	0.0789	0.0659
April '07	0.7000	0.4693	0.0896	0.0783
May '07	0.6000	0.4326	0.1078	0.0965
Jun '07	0.6500	0.4598	0.0789	0.0659
July '07	0.1250	0.1187	0.1078	0.0965
August '07	0.0750	0.0731	0.1078	0.0965
September '07	0.6000	0.4326	0.0896	0.0783
October '07	0.7500	0.4924	0.0896	0.0783
November '07	0.8750	0.4984	0.0789	0.0659
December '07	0.9250	0.5035	0.1078	0.0965

3.1.3. Polymorphism at *Pgm* and *Hk* Loci in Hatay

(*A. mellifera syriaca*) Population

A total number of 560 (14 month ×40 individuals/month) worker bees were studied in the electrophoretic analysis of *Pgm* and *Hk* loci of samples collected from Hatay.

Among the four alleles observed (*Pgm-65, Pgm-75, Pgm-100, Pgm-110*), *Pgm-65* and *Pgm-110* were the rare alleles having the smallest frequencies both in the total number of bees analyzed (Table 3.15) and in each of the 14 months from November 2006 to December 2007 (Table 3.17). Alleles *Pgm-75* and *Pgm-100* comprise the 99.48 % of all alleles with *Pgm-75* being the common allele in Hatay samples as in the case of the Kırklareli and Artvin populations.

Table 3.15. Distribution of the observed *Pgm* alleles in Hatay samples.

Allele	Count	Percentage
Pgm-65	3	0.26
Pgm-75	717	71.18
Pgm-100	317	28.3
Pgm-110	3	0.26
TOTAL	1120	100.0

Pgm 75/100 and *Pgm-75/75* had the highest frequency together comprising the 99.1% of the all genotypes observed over 14 months for Hatay population.

Table 3.16. Distribution of the observed *Pgm* alleles in Hatay samples.

Genotype	Count	Percentage
Pgm-*75/100*	300	53.57
Pgm-*75/75*	255	45.53
Pgm-*100/100*	0	0
Pgm-*65/75*	2	0.35
Pgm-65/100	0	0
Pgm 75/110	3	0.55
TOTAL	560	100

Table 3.17. Allele frequencies at *Pgm* and *Hk* loci for each month from November 2006 to December 2007 for Hatay (*A.mellifera syriaca*) population.

Sample Collection month and year (n=40/ month)	**ALLELE FREQUENCIES**					
	Pgm-75	***Pgm-100***	***Pgm-65***	***Pgm-110***	***Hk-100***	***Hk-87***
November '06	0.60	0.40	0.00	0.00	0.90	0.10
December '06	0.525	0.465	0.00	0.01	0.90	0.10
January '07	0.525	0.475	0.00	0.00	0.875	0.125
February '07	0.525	0.475	0.00	0.00	0.925	0.075
March '07	0.537	0.463	0.00	0.00	0.95	0.05
April '07	0.80	0.19	0.01	0.00	0.90	0.10
May '07	0.83	0.15	0.00	0.02	0.925	0.075
Jun '07	0.93	0.06	0.01	0.00	0.95	0.05
July '07	0.94	0.06	0.00	0.00	0.925	0.075
August '07	0.96	0.04	0.00	0.00	0.925	0.075
September '07	0.84	0.16	0.00	0.00	0.875	0.125
October '07	0.84	0.16	0.00	0.00	0.90	0.10
November '07	0.71	0.29	0.00	0.00	0.875	0.125
December '07	0.62	0.38	0.00	0.00	0.95	0.05

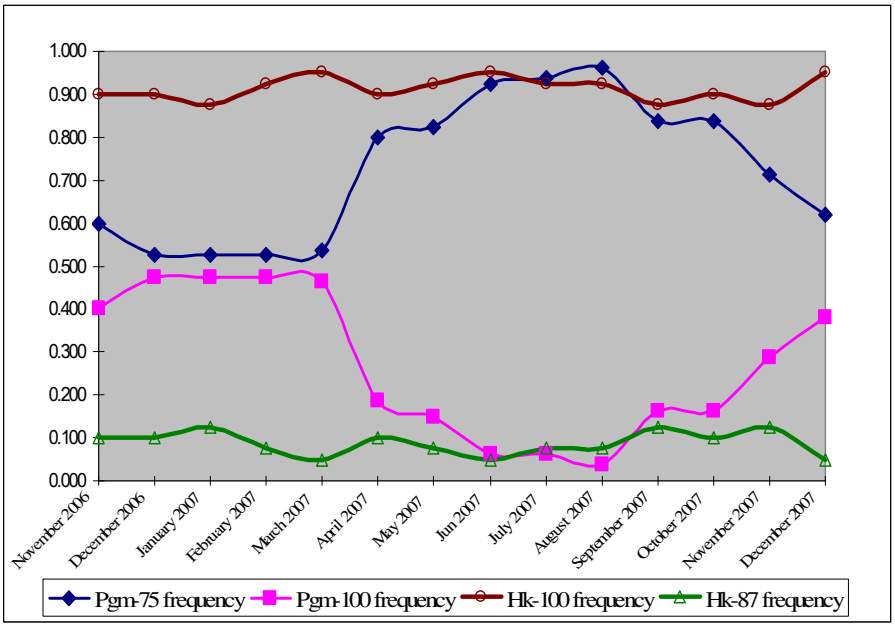

Figure 3.7. Change in allele frequencies of *Pgm* and *Hk* loci over 14 months in Hatay (*A. mellifera syriaca*) samples.

Alleles observed at *Hk* locus in Hatay region were the same as in the analysis of Kırklareli and Artvin samples: *Hk-87* and *Hk-100* (frequencies of alleles given in Table 3.17). *Hk-100* was the most common allele in this locus. The genotypes observed were: *Hk-87/100* and *Hk-100/100* and their frequencies for each month are given in Table 3.20. There were no significant difference in the *Hk* allele and genotype frequencies between months or seasons and genotype frequencies were found to be in Hardy-Weinberg equilibrium regardless of the time of sample collection (Table 3.17 - Table 3.20). The temporal stability of *Hk* allele and genotype frequencies are illustrated in Figure 3.7 and Figure 3.8 respectively.

In contrast to the seasonal stability of *Hk* allele and genotype frequencies, summer and winter bees differed significantly ($P < 0.001$) in terms of their frequency of *Pgm* alleles (*Pgm-75* and *Pgm-100*). The genotype frequencies of the samples collected in

summer were found to be in Hardy-Weinberg equilibrium in contrast to the winter samples which showed significant levels of deviations. The comparisons of allele frequencies of winter and summer samples are tabulated in Table 3.17 for each month. The deviations of the genotype frequencies from the equilibrium were tested by likelihood ratio test (G^2) and the results are given in Table 3.18.

Table 3.18. Frequencies of *Pgm 75/75* and *Pgm 75/100* over 14 months for Hatay with their corresponding G^2 and *P values.*

Sample collection month and year (n=40 / Month)	**GENOTYPE FREQUENCIES**			
	Pgm-75/100	***Pgm-75/75***	***G^2***	***P***
November.'06	0.8	0.2	9.146	0.002
December' 06	0.9	0.075	28.76	0.000
January '07	0.95	0.05	41.23	0.000
February '07	0.95	0.05	41.23	0.000
March' 07	0.925	0.075	36.98	0.000
April' 07	0.375	0.6	3.792	0.284
May' 07	0.3	0.65	2.779	0.143
Jun '07	0.125	0.85	0.405	0.939
July '07	0.125	0.875	0.266	0.605
August '07	0.075	0.925	0.077	0.780
September '07	0.325	0.675	2.344	0.125
October '07	0.325	0.675	2.344	0.125
November '07	0.575	0.425	9.146	0.002
December '07	0.75	0.25	18.67	0.000

Table 3.19. Frequencies rare genotypes at *Pgm* locus for each month from for Hatay (*A. mellifera syriaca*) population.

Sampling month and year (n=40 /Month)	**GENOTYPE FREQUENCIES**			
	Pgm-100/100	***Pgm-65/75***	***Pgm-65/100***	***Pgm-75/110***
November.'06	0.00	0.00	0.00	0.00
December' 06	0.00	0.00	0.00	0.025
January '07	0.00	0.00	0.00	0.00
February '07	0.00	0.00	0.00	0.00
March' 07	0.00	0.00	0.00	0.00
April' 07	0.00	0.025	0.00	0.00
May' 07	0.00	0.00	0.00	0.05
Jun '07	0.00	0.025	0.00	0.00
July '07	0.00	0.00	0.00	0.00
August '07	0.00	0.00	0.00	0.00
September '07	0.00	0.00	0.00	0.00
October '07	0.00	0.00	0.00	0.00
November '07	0.00	0.00	0.00	0.00
December '07	0.00	0.00	0.00	0.00

Table 3.20. Genotype frequencies at *Hk* locus over 14 months for Hatay.

Sampling month and year (n=40 / Month)	**GENOTYPE FREQUENCIES**		
	Hk-100/100	***Hk-87/100***	***P***
November '06	0.8	0.2	0.670
December '06	0.8	0.2	0.670
January '07	0.75	0.25	0.645
February '07	0.85	0.15	0.780
March '07	0.9	0.1	0.869
April '07	0.8	0.2	0.670
May '07	0.85	0.15	0.780
Jun '07	0.9	0.1	0.869
July '07	0.85	0.15	0.780
August '07	0.85	0.15	0.780
September '07	0.75	0.25	0.645
October '07	0.8	0.2	0.670
November '07	0.75	0.25	0.645
December '07	0.9	0.1	0.869

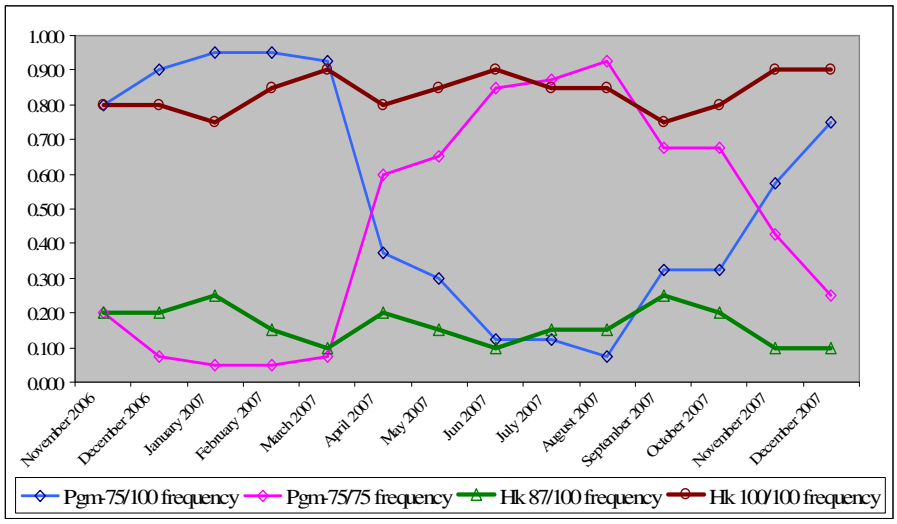

Figure 3.8. Change in genotype frequencies of *Pgm* and *Hk* loci over 14 months in Hatay (*A. mellifera syriaca*) samples.

Table 3.21. Observed and expected heterozygosity values for *Pgm* and *Hk* loci for each month from November 2006 to December 2007 for Hatay population.

Sample Collection month and year (n=40 / month)	**Observed and Expected Heterozygosity Values**			
	Observed *-Pgm-*	**Expected *-Pgm-***	**Observed *-Hk-***	**Expected *-Hk-***
November '06	0.7546	0.7498	0.0789	0.0659
December '06	0.7856	0.5603	0.0789	0.0659
January '07	0.8000	0.4861	0.1267	0.1145
February '07	0.9250	0.5149	0.0789	0.0659
March '07	0.9048	0.4563	0.0896	0.0783
April '07	0.5845	0.5545	0.1078	0.0965
May '07	0.3243	0.2755	0.0789	0.0659
Jun '07	0.1500	0.1403	0.0896	0.0896
July '07	0.1250	0.1187	0.0789	0.0659
August '07	0.0750	0.0731	0.0789	0.0659
September '07	0.3250	0.2756	0.1267	0.1145
October '07	0.3250	0.2756	0.0789	0.0659
November '07	0.5750	0.4149	0.1267	0.1145
December '07	0.7500	0.4747	0.0896	0.0783

The heterozygosity values at *Pgm* and *Hk* loci of Hatay samples computed for each month are given in Table 3.21. At *Pgm* locus, samples collected in winter months had mean heterozygosity of 0.935 and for the samples collected in summer months the average heterozygosity is 0.143. The frequency of the Pgm-*75/100* heterozygotes differed significantly between summer and winter months ($P < 0.001$). There was no significant seasonal difference in heterozygosity at Hk locus; heterozygosity remained stable throughout the year.

3.2. Analysis of Protein Measurements

Absorbance readings (570nm) of samples were used for the determination of protein concentrations from their corresponding standard curves. Protein measurements were performed for the standardization of PGM enzyme activity assays and glycogen measurements of samples so that the results obtained from these assays become comparable. The mean of all samples (n=40) is 9.448. Among the 4 block of 10 samples no significant difference was observed in terms of their protein content. Also, there was no significant difference in the protein content of winter and summer bees. Results are given in Table 3.22 and Table 3.23.

Table 3.22. Protein content of winter samples with their corresponding genotypes, means and standard deviations.

1st Block	Genotype	[Protein] mg/mL	2nd Block	Genotype	[Protein] mg/mL
1	*Pgm 75/100*	8.1	1	*Pgm 75/100*	8.5
2	*Pgm 75/100*	7.03	2	*Pgm 75/100*	9.3
3	*Pgm 75/100*	6.9	3	*Pgm 75/100*	10.2
4	*Pgm 75/100*	10.5	4	*Pgm 75/100*	11.3
5	*Pgm 75/100*	9.8	5	*Pgm 75/100*	9.1
6	*Pgm 75/100*	8.9	6	*Pgm 75/100*	8.8
7	*Pgm 75/75*	9.5	7	*Pgm 75/100*	11.2
8	*Pgm 75/100*	11.3	8	*Pgm 75/100*	9.6
9	*Pgm 75/100*	9.2	9	*Pgm 75/100*	7.9
10	*Pgm 75/100*	10.6	10	*Pgm 75/100*	7.3
Mean= 9.18, SD= 1.48			**Mean= 9.32, SD= 1.30**		

Table 3.23. Protein content of summer samples with their corresponding genotypes, means and standard deviations.

3rd Block	Genotype	[Protein] mg/mL	4th Block	Genotype	[Protein] mg/mL
1	*Pgm 75/75*	8.6	1	*Pgm 75/75*	7.5
2	*Pgm 75/75*	9.1	2	*Pgm 75/100*	9.8
3	*Pgm 75/75*	7.7	3	*Pgm 75/75*	10.2
4	*Pgm 75/75*	8.9	4	*Pgm 75/75*	10.6
5	*Pgm 75/100*	9.3	5	*Pgm 75/75*	11.3
6	*Pgm 75/75*	11.5	6	*Pgm 75/75*	9.5
7	*Pgm 75/75*	10.9	7	*Pgm 75/75*	8.9
8	*Pgm 75/75*	11.2	8	*Pgm 75/100*	9.2
9	*Pgm 75/75*	7.6	9	*Pgm 75/75*	10.7
10	*Pgm 75/75*	8.3	10	*Pgm 75/75*	12.1
Mean= 9.31, SD= 1.42			**Mean= 9.68, SD= 1.30**		

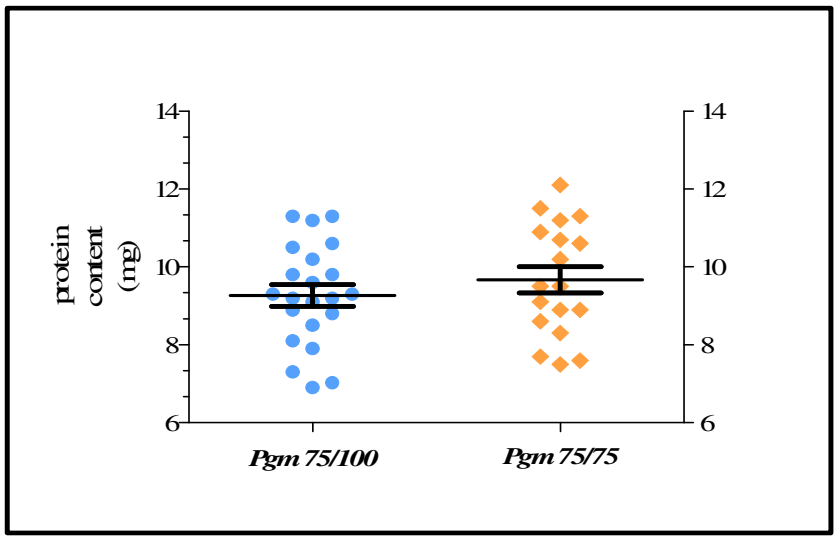

Figure 3.9. The distribution of the protein content of the samples around their group means.

3.3. PGM Enzyme Activity Analysis

Enzyme activity measurements were performed with 4 blocks of 10 samples composed of bees collected both in summer and winter months. The genotypes of the samples used in enzyme activity assays were determined after glycogen and protein measurement assays were also completed with the same individuals.

Each sample was assayed individually for the determination of the PGM enzyme activity and the calculations were made according to the formula:
µmol of NADP reduced/min/ml = (ΔA-340 nm/min) X (1 ml assay volume/6.2) X (1/0.055 ml extract volume) X (dilution factor). Both the activity in assay solution and specific activity standardized by the protein amount is given for winter and summer bees in Tables 3.24, 3.25, 3.26, 3.27.

Table 3.24. PGM activities of the winter samples in the first block of assay with their corresponding genotypes.

sample	**PGM activity (units/mL)**	**PGM activity units/mg protein**	**Genotype**
1	25.27	3.12	*Pgm 75/100*
2	23.12	3.29	*Pgm 75/100*
3	20.83	3.02	*Pgm 75/100*
4	33.70	3.21	*Pgm 75/100*
5	29.20	2.98	*Pgm 75/100*
6	27.59	3.10	*Pgm 75/100*
7	19.28	2.03	*Pgm 75/75* *
8	34.91	3.09	*Pgm 75/100*
9	31.00	3.37	*Pgm 75/100*
10	34.23	3.23	*Pgm 75/100*

Table 3.25. PGM activities of the winter samples in the second block of assay with their corresponding genotypes.

Sample	**PGM activity (units/mL)**	**PGM activity units/mg protein**	**Genotype**
1	25.16	2.96	*Pgm 75/100*
2	28.73	3.09	*Pgm 75/100*
3	32.64	3.2	*Pgm 75/100*
4	36.047	3.19	*Pgm 75/100*
5	29.57	3.25	*Pgm 75/100*
6	25.6	2.91	*Pgm 75/100*
7	34.49	3.08	*Pgm 75/100*
8	31.39	3.27	*Pgm 75/100*
9	24.727	3.13	*Pgm 75/100*
10	25.98	3.56	*Pgm 75/100*

Table 3.26. PGM activities of the summer samples in the third block of assay with their corresponding genotypes.

Sample	PGM activity (units/mL)	PGM activity units/mg protein	Genotype
1	16.68	1.94	*Pgm 75/75*
2	16.92	1.86	*Pgm 75/75*
3	15.631	2.03	*Pgm 75/75*
4	16.91	1.9	*Pgm 75/75*
5	32.92	3.54	*Pgm 75/100* *
6	21.275	1.85	*Pgm 75/75*
7	22.454	2.06	*Pgm 75/75*
8	21.39	1.91	*Pgm 75/75*
9	13.6	1.79	*Pgm 75/75*
10	16.09	1.93	*Pgm 75/75*

Table 3.27. PGM activities of the summer samples in the fourth block of assay with their corresponding genotypes.

sample	PGM activity (units/mL)	PGM activity units/mg protein	Genotype
1	15.52	2.07	*Pgm 75/75*
2	32.536	3.32	*Pgm 75/100* *
3	19.73	1.97	*Pgm 75/75*
4	21.41	2.02	*Pgm 75/75*
5	20.56	1.82	*Pgm 75/75*
6	17.67	1.86	*Pgm 75/75*
7	18.69	2.1	*Pgm 75/75*
8	29.53	3.21	*Pgm 75/100* *
9	21.93	2.05	*Pgm 75/75*
10	21.65	1.79	*Pgm 75/75*

Unpaired, two-tailed *t* test was used to determine whether the observed enzyme activity difference between *Pgm-75/75* and *Pgm-75/100* genotypes is statistically significant. The homozygote and heterozygote individuals were pooled into separate groups to determine whether two groups differ in terms of their PGM activity. The result of the unpaired *t*-test is given in Table 3.28.

Table 3.28. Result of the unpaired *t* test for enzyme activities of *Pgm-75/75* and *Pgm-75/100* genotypes ($P < 0.0001$, $t=27.69$, $df= 38$, standard error of difference = 0.045).

Group	***Pgm-75/75***	***Pgm-75/100***
Mean	1.9433	3.1873
Standard deviation	0.1015	0.1668
Sample size	18	22

PGM activity is significantly higher in heterozygote (*Pgm-75/100*) individuals ($P < 0.0001$) whose frequency increase during the winter months.

3.4. Analysis of Glycogen Measurements

Glycogen contents of both summer and winter bees were calculated from the standard curves of their absorbance readings. The lowest amount of glycogen was 0.87 (mg/mg protein) from a summer bee and the highest glycogen amount was 2.67 (mg/mg protein) from a winter bee (Table 3.29 and Table 3.30). The glycogen contents of the winter and summer samples, means and standard deviations of the groups are given in Table 3.29 and Table 3.30.

Comparison of the glycogen content of the summer and winter bees was done by using unpaired, two-tailed *t* test after *Pgm-75/75* homozygotes and *Pgm-75/100* heterozygotes were pooled into two groups (Table 3.31).

Table 3.29. Glycogen content of the winter samples with their corresponding genotypes.

1st block	**Genotype**	**[Glycogen] mg/mg protein**	**2nd block**	**Genotype**	**[Glycogen] mg/mg protein**
1	*Pgm 75/100*	2.3	1	*Pgm 75/100*	2.19
2	*Pgm 75/100*	2.49	2	*Pgm 75/100*	2.3
3	*Pgm 75/100*	2.43	3	*Pgm 75/100*	2.35
4	*Pgm 75/100*	2.36	4	*Pgm 75/100*	2.21
5	*Pgm 75/100*	2.2	5	*Pgm 75/100*	2.39
6	*Pgm 75/100*	2.3	6	*Pgm 75/100*	2.1
7	*Pgm 75/75*	1.05	7	*Pgm 75/100*	2.29
8	*Pgm 75/100*	2.27	8	*Pgm 75/100*	2.41
9	*Pgm 75/100*	2.52	9	*Pgm 75/100*	2.32
10	*Pgm 75/100*	2.47	10	*Pgm 75/100*	2.67
Mean= 2.23, SD= 0.43			**Mean= 2.32, SD= 0.154**		

Table 3.30. Glycogen content of the summer samples with their corresponding genotypes.

3rd block	**Genotype**	**[Glycogen] mg/mg protein**	**4th block**	**Genotype**	**[Glycogen] mg/mg protein**
1	*Pgm 75/75*	1.69	1	*Pgm 75/75*	1.65
2	*Pgm 75/75*	0.97	2	*Pgm 75/100*	2.41
3	*Pgm 75/75*	1.12	3	*Pgm 75/75*	1.54
4	*Pgm 75/75*	1.59	4	*Pgm 75/75*	1.65
5	*Pgm 75/100*	2.59	5	*Pgm 75/75*	1.05
6	*Pgm 75/75*	1.07	6	*Pgm 75/75*	1.1
7	*Pgm 75/75*	1.19	7	*Pgm 75/75*	1.53
8	*Pgm 75/75*	1.51	8	*Pgm 75/100*	2.3
9	*Pgm 75/75*	0.89	9	*Pgm 75/75*	1.23
10	*Pgm 75/75*	1.6	10	*Pgm 75/75*	0.87
Mean= 1.422, SD= 0.52			**Mean= 1.533, SD= 0.50**		

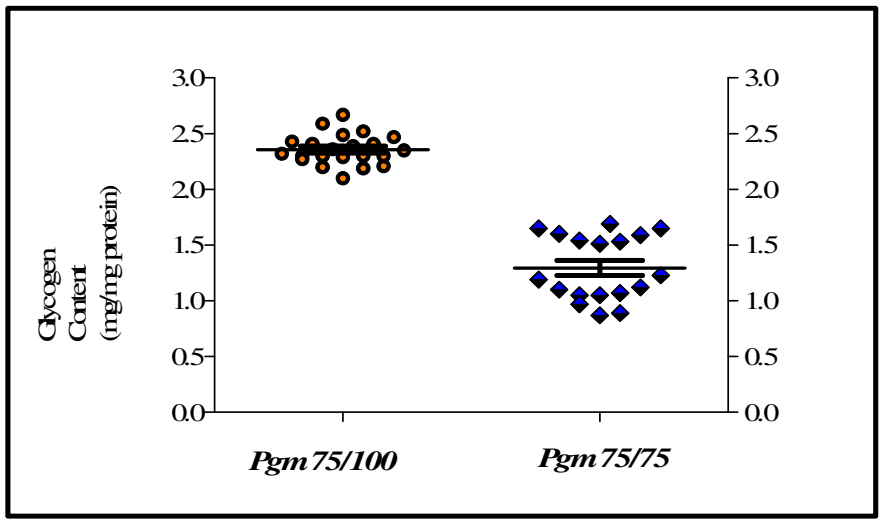

Figure 3.10. The distribution of the glycogen content of the samples *Pgm-75/100* and *Pgm-75/75* genotypes around their means.

Table 3.31. Result of the unpaired *t* test for glycogen content of *Pgm-75/75* and *Pgm-75/100* genotypes ($P < 0.0001$, t=15.39, df= 38, standard error of difference = 0.070).

Group	***Pgm-75/75***	***Pgm-75/100***
Mean	1.2944	2.3577
Standard deviation	0.2925	0.1359
Sample size	18	22

Glycogen content of the *Pgm-75/100* heterozygotes was significantly higher than the *Pgm-75/75* homozygotes ($P < 0.0001$). Figure 3.11 and Figure 3.12 show the glycogen content and enzyme activity of the heterozygote and homozygote individuals respectively.

Glycogen content is significantly higher in heterozygote individuals which have high PGM enzyme activity. To determine whether the increased glycogen content of the heterozygotes is correlated with enzyme activity a non-parametric test (Spearman test) was performed. It was determined that there was a significant correlation between the enzyme activity and glycogen content (Spearman r=0.8410, $P<0.0001$) for heterozygote individuals (Figure 3.13). Correlation between enzyme activity and glycogen content (Figure 3.14) was found also in homozygote individuals (Spearman r=0.5665, $P= 0.0142$). Test of homogeneity of the correlation coefficients of enzyme activity vs. glycogen content of two groups (heterozygote and homozygote individuals) revealed that there is no significant difference between correlations ($\chi^2= 2.70$, $P= 0.1$). Therefore the difference in glycogen content of the heterozygotes and homozygotes result from the high PGM enzyme activity of the heterozygote individuals.

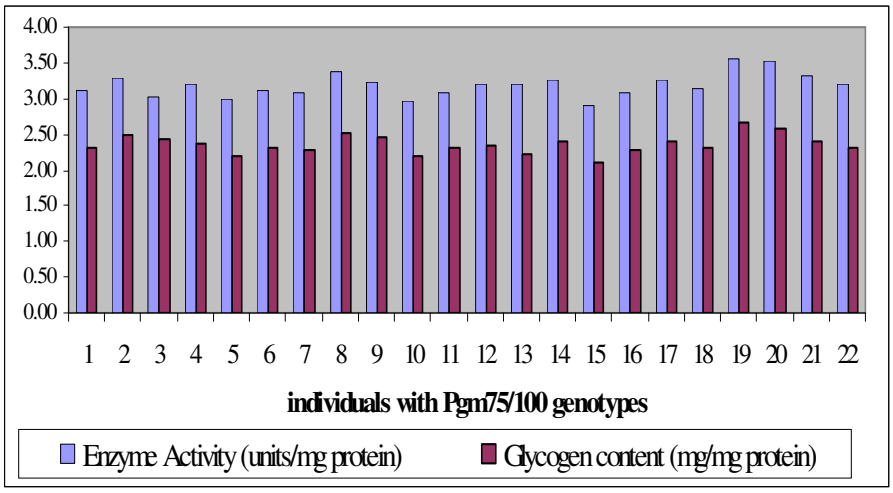

Figure 3.11. PGM activity and glycogen content of individuals with *Pgm-75/100* genotypes.

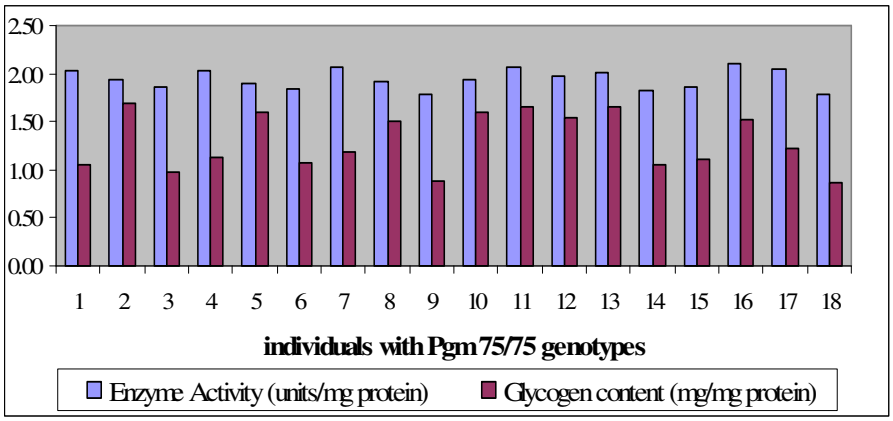

Figure 3.12. PGM activity and glycogen content of individuals with *Pgm-75/75* genotypes.

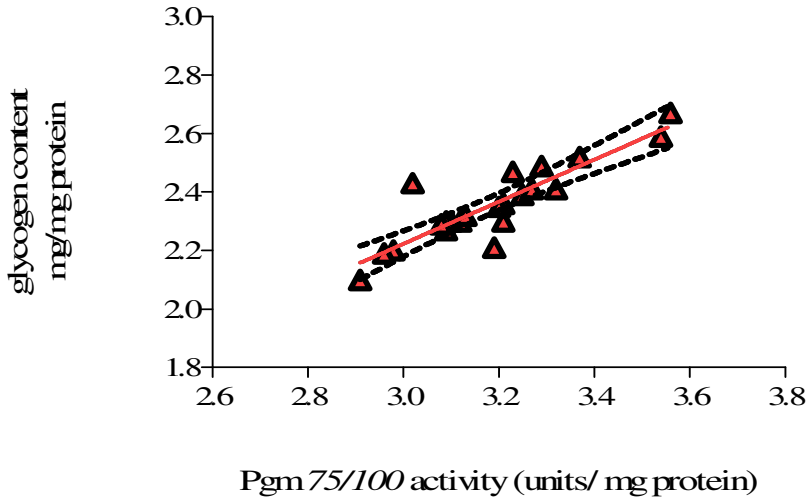

Figure 3.13. Correlation between *Pgm 75/100* enzyme activity and glycogen content (Confidence interval 0.95 illustrated by dotted line Spearman r=0.8410, *P*<0.0001)

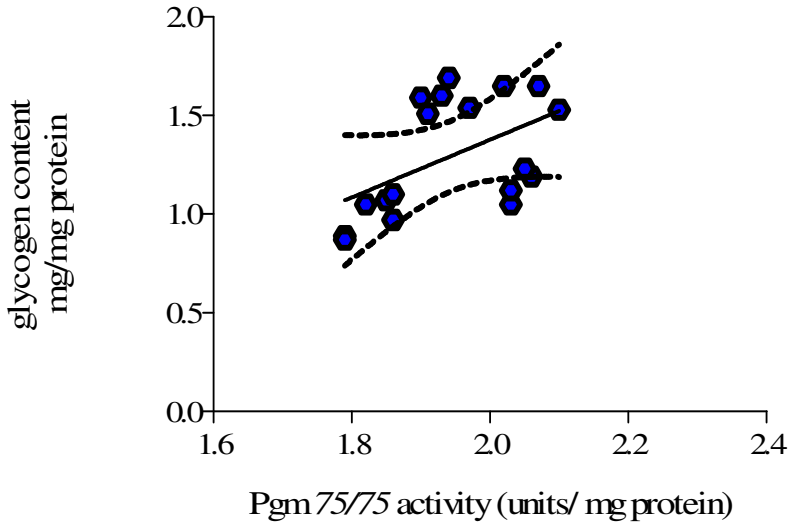

Figure 3.14. Correlation between *Pgm 75/75* enzyme activity and glycogen content (Confidence interval 0.95 illustrated by dotted line Spearman r=0.5665, *P*= 0.0142)

CHAPTER 4

DISCUSSION

This is the first study that demonstrates the seasonal variation of *Pgm* polymorphism in three subspecies of honeybee (*Apis mellifera* L.) with its some functional and physiological correlates. Although earlier studies have provided information regarding the presence of seasonal change in allele frequencies at *Pgm* locus regarding one subspecies (Hadimoğulları *et al*., 2002), long time analysis with regular sampling over seasons have not been performed to follow the frequency changes. Monthly analysis of *Pgm* polymorphism from three ecologically different locations enabled the characterization of the seasonal variation by determination of the allele frequency peaks and drops in response to changes in temperature. Furthermore, biochemical analysis of the different genotpes of *Pgm* have revealed that heterozygotes (*Pgm-75/100*) had significantly higher levels of enzyme activity than the homozygotes (*Pgm-75/75*) meaning that enzyme variants are functionally different. There is also a significant positive correlation between the enzyme activity of different *Pgm* genotypes and glycogen content. Therefore, the results of this study support the idea that not all protein polymorphisms are neutral and some can underlie functional differences. These differences may have adaptive value since biochemical differences in the allelic variants of a single enzyme locus have the potential to influence physiological processes.

4.1. Seasonal Variation at Pgm Locus in honeybees

One major finding in this study is that *Pgm* genotypes cycle through the year in independent populations at different locations belonging to three subspecies namely *A. mellifera caucasica*, *A.m.syriaca*, and A.*m.carnica*. Results obtained from the allozyme analysis of *Pgm* and *Hk* loci on the samples collected at regular periods from three locations (Kırklareli, Hatay, and Artvin) have given similar patterns of seasonal shifts in the allele and genotype frequencies of *Pgm.* The *Pgm* heterozygote frequency increases during autumn months, peaks in winter and drops in spring, stays low through summer. Whereas, *Hk* locus studied as the control maintained a stable frequency of genotypes throughout the year.

No significant variation was observed between the localities in terms of the pattern of the cyclic seasonal variation of genotype and allele frequencies at *Pgm* locus although the ecological characteristics of the sampling sites differ from each other significantly in terms of average temperature and precipitation. The declines and peaks in the frequency of allele frequencies of *Pgm* occurred at similar times in all locations. At the end of the summer, in September, *Pgm-75/100* frequency started to increase rapidly and the increase continued until April with simultaneous decline in *Pgm-75/75* frequency. Arrival of the spring caused a rapid drop in *Pgm-75/100* frequency which is accompanied by the peak in the frequency of *Pgm-75/75* homozygotes. Throughout the summer homozygotes (*Pgm-75/75*) maintained high frequencies (average in the range of 0.86-0.90 in all locations), as heterozygotes maintained average frequencies of 0.92 or even 0.95 in winter months. The obvious difference in *Pgm-75/100* and *Pgm-75/75* genotype frequencies between summer and winter months reflected itself with statistically significant deviations from the expected heterozygosity values in summer months. Frequencies of *Pgm-75* and *Pgm-100* alleles followed a similar pattern of cyclical changes through seasons. The

genotype frequencies of the samples collected in summer were found to be in Hardy-Weinberg equilibrium in contrast to the winter samples which showed significant levels of deviations ($P < 0.001$). Whereas, there was no significant difference in genotype frequencies for the *Hk* locus, *Hk* genotype and allele frequency was stable in all locations independent of the time of sample collection.

It is apparent that not all but some enzyme loci respond to the seasonal fluctuations in ecological parameters. In this study, in contrast to the cyclical changes of allele and genotype frequencies at *Pgm* locus, no such pattern was observed for the *Hk* alleles. The occurrence of such pattern of seasonal variations in allele frequencies of an enzyme locus can significantly alter the results of studies concerned with the comparisons of allozyme data from geographically distinct locations. Erroneous conclusions can be drawn if allele frequencies at enzyme loci are assumed to be constant through time. The assumption that allozyme frequencies are stable through seasons and thus allozyme data can be compared among different locations need to be revised once again since results of the studies that focus on changes in allelic frequencies demonstrate the occurrence of temporal variations in allele frequencies of natural populations. Accordingly, as the results of this study has shown, the allele and genotype frequencies at *Pgm* locus should be given together with the time of the year or temperature conditions of the locality in honey bees and perhaps in other organisms. In other words, without the information of sample collection time and climatic characteristics of the location, data of *Pgm* allele and genotype frequencies may represent inaccurate estimates of population structure parameters. For example, heterozygosity estimates of a honeybee population sampled in winter will have higher values than a population sampled in summer or spring. Therefore, comparison of the allele frequencies of *Pgm* from individuals sampled at different times of the year may be misleading that is may cause either over or underestimation of the population parameters. This should be considered for other enzymes as well.

A general criticism for studies of temporal variations in allele and genotype frequencies at enzyme loci have been low sample sizes, implying that the observed temporal variations at an enzyme loci may result from inadequate sampling and

consequent observation of cyclical changes resulting from random fluctuations (Cavener and Clegg, 1981). In this study a total number of 2540 honeybees were analyzed and a whole year survey of *Pgm* and *Hk* loci was completed for all locations studied. The data clearly demonstrated the regular seasonal shifts in allele and genotype frequencies at *Pgm* locus in honeybees belonging to different subspecies and locations.

Several hypotheses could be formulated to explain differences in *Pgm* allele frequencies over the year. An hypothesis is that *Pgm* may be linked to the expression of heat-shock protein producing genes and contribute to tolerance to temperature changes; Neargarder *et al*. (2003) reported such a linkage with *Pgi* in mountane leaf beetle, *Chrysomella aeneicollis*. It has been determined that the *Pgi* genotypes differed in heat-shock protein expression profiles which in turn led to functional and physiological differences among *Pgi* genotypes which significantly affect the survival of the organisms during exposure to extreme temperatures (Dahlhoff and Rank, 2000; Neargarder *et al*. 2003). Similarly, seasonal changes in *Pgm* allele frequencies in honeybees may result from the differential expression of regulatory loci that control the expression of the different *Pgm* alleles in response to the changes in temperature. Activation and deactivation of the regulatory loci during winter and summer may be the reason for the corresponding increase and decline in *Pgm* allele frequencies over the seasons. Accordingly, observed allozyme differences at *Pgm* locus may be mere reflection of altered gene expression, as seen in the brains of foragers and nurses that occur in response to queen mandibular pheromone (Grozinger *et al*., 2003).

Another explanation may be that the killing of young homozygous individuals by workers present in the colony prior to winter season. *Pgm* homozygotes may consume more honey compared to heterozygote individuals during winter. The amount of honey in colony is very important for the survival of the colony during winter and cannibalism of larvae that are homozygotes for *Pgm* may be a strategy employed by honeybees for more efficient consumption of honey to ensure winter survival. There are examples of mechanisms of phenotypic assessment of genotypes

in social insects by workers, followed by elimination of undesired genotypes such as killing of the diploid drone larvae by the worker bees that eat them (Woyke, 1963; 1976; Schmickl and Crailsheim, 2001). It has been reported that cannibalism behavior of the workers is significantly affected by both environmental factors such as pollen and nectar availability, weather conditions and also by the intracolonial conditions so that reduction in the availability of the pollen caused earlier sealing of the brood cells by workers in the hive and cannibalism of the young larvae to cope with the shortage of pollen supply (Schmickl and Crailsheim, 2002).

Pgm-75/75 frequency declined rapidly with the arrival of the autumn in September, therefore, decreased food availability and poor weather conditions may trigger the cannibalism of the larvae that are somehow detected as *Pgm* homozygotes by workers.

All of these hypotheses were based primarily on the novel phenomenon observed, *Pgm* genotype frequency changes over the seasons to explain how changes in seasonal ecological conditions can reflect themselves in honeybee colonies. However, based merely on the *Pgm* allozyme data, without considering the biochemical and physiological correlates of *Pgm* polymorphism, it is hard to describe a reasonable mechanism that can estimate the one employed by honeybees over seasons. Correlation of different *Pgm* genotypes with PGM enzyme activity and glycogen measurements may facilitate the evaluation of the difference in *Pgm* expression patterns between summer and winter bees.

4.2. Biochemical and Physiological Correlates

Analysis of enzyme activity of *Pgm-75/100* and *Pgm-75/75* genotypes revealed that enzyme activity is significantly higher in heterozygote individuals ($P<0.0001$). The mean PGM activity measured for homozygotes was 1.943 units/ mg protein and for heterozygotes 3.187 units/ mg protein. Moreover, summer and winter bees differed

significantly in terms of their glycogen content (P<0.0001). Glycogen content is significantly higher in heterozygote individuals which have high levels of PGM enzyme activity. Furthermore, it was observed that PGM activity is an important determinant of glycogen content of the bees since enzyme activity and glycogen content was found to be significantly correlated (Spearman r =0.8410, P<0.0001). Therefore, enzyme activity and glycogen content of the bees were found to follow a similar seasonal pattern as in the case of the shifts in heterozygosity that is highly dependent on the allelic composition at the *Pgm* locus. As an important branch point enzyme in glycolytic pathway and the pentose shunt, it is evident that genetic differences at *Pgm* locus can lead to functional variations in terms of enzyme activity which in turn can influence energy metabolism and glycogen content of honeybees.

Although, the "theory of metabolic flux" (Kacser and Burns, 1981) hypothesizes that enzyme polymorphisms and biochemical variations in enzyme variants can not lead to detectable changes in the metabolic flux and electrophoretically different enzymes are functionally identical, it was observed in the present study that significant variations in enzyme activity of the loci that are central to the metabolism have the potential to alter the metabolic output as predicted by several other authors (Johnson, 1973; Watt, 1985; Eanes, 1999; Verrelli and Eanes, 2001b).

In addition to the regulatory role of PGM in energy metabolism; directing the flux through glycogen synthesis or degradation depending on the needs of the organism, the fact that glycogen reserves are the main source of energy for honeybees may contribute to the observed significant association between enzyme activity and glycogen amount. In other words, the correlation may not be so significant if honeybees were dependent on other metabolites such as lipids or proteins for energy production. A possible mechanism that is suggested to explain the positive correlation between PGM enzyme activity and glycogen concentrations in *Drosophila melanogaster* is that the concentration of the glucose-6-phosphate may cause inhibition or activation of other enzymes in the glycolytic pathway due to competition with these branches resulting in the increased flux through glycogen synthesis (Verrelli and Eanes, 2001b). Although the mechanism directing the flux

remains to be solved, analysis of biochemical correlates of the genetic variation at *Pgm* locus in honeybees, as well as the other enzyme loci studied in different organisms (Watt 1983, 1992, 1994; Hoffman, 1985; Dahlhoff and Rank, 2000; Verrelli and Eanes, 2001b) suggest that enzyme polymorphisms can influence metabolic pathway characteristics. Furthermore, characterization of different *Pgm* electrophoretic variants by measurements of enzyme kinetics and thermostability parameters have revealed that, differerent genotypes show variations in terms of their affinity for the glucose-1-P which in turn affected the survival and growth of the larvae of the *D. melanogaster* growing under different concentrations of glucose indicating that individuals having different *Pgm* genotypes show variations in their metabolic utilization of glygogen reserves (Fucci et al., 1979).

The observation that heterozygote individuals that occur in high frequencies during winter months had both higher levels of PGM activity and glycogen content as compared to homozygotes whose frequency increase during summer must be evaluated by considering the several factors most of which are unique to honeybees. Among these factors, different life span of the winter and summer bees is of particular importance since the longer life span of the winter bees as compared to summer bees ensure the survival of the broodless colony during winter (Matilla and Otis, 2007). It has been reported that the main factor affecting the life span of honeybees is the duration of the "hive period" that is the period that the bees perform tasks inside the hive without foraging and flight performance during foraging (Neukirch, 1982). Studies have demonstrated that increased life span of the winter bees is mainly due to the increased "hive-period" and relatively shorter life span of summer bees result from the extensive periods of flight (Neukirch, 1982). Furthermore, reduced glycogen amount and synthesis was found to increase mortality of the worker bees that expend high amounts of energy during foraging (Neukirch, 1982). These findings suggest that glycogen content in honeybees is significantly associated with life span and indicate that the reduction in glycogen reserves of the summer bees due to high expenditures of energy during foraging period is the reason for their short life span.

However, like foraging in summer, the hive-period of the winter bees involves high demands of metabolic energy in order to increase and maintain the temperature inside the hive by social thermoregulation. It has been reported that the metabolic cost of thermoregulation by forming winter clusters inside the hive is similar to the cost associated with foraging flights also metabolic rate and glycogen reserves of foragers and cluster bees were found to be similar (Panzenbock and Crailsheim, 1997).

Therefore, it should be noted that the longevity of the winter bees are not due to the low metabolic rates since they also consume the glycogen reserves as summer bees do during foraging to compensate the metabolic cost associated with thermoregulation. Then, there should be another reason for the high glycogen content and longer life span of the winter bees although both the summer and winter bees use glycogen reserves at similar metabolic rates to compensate similar metabolic costs.

The observation that *Pgm* heterozygotes that predominate in winter colonies independent of geographical location, have both high levels of PGM activity and glycogen content may be the underlying mechanism to the increased life span during winter because increased PGM activity may provide efficient utilization of the glycogen reserves as compared to homozygotes which occur in high frequencies during summer. Therefore increased heterozygosity at *Pgm* locus during winter periods can be an important physiological mechanism that contributes to the efficient use of the glycogen reserves for production of heat during thermoregulation and longevity of the bees which is important for the survival of the broodless colony from autumn to the next spring. Increased biochemical efficiency of heterozygote individuals compared to that of homozygotes is a common mechanism associated with enzyme polymorphisms. Mitton (1997) has suggested that on one hand, the increased biochemical efficiency of the heterozygote individuals result from the expression of alleles that produce enzymes with more efficient catalytic properties, on the other hand homozygotes can have only a single form of the enzyme. Also, it is possible that more efficient use of the glycogen reserves and metabolic energy by

Pgm heterozygotes may allow allocation of more energy for other metabolic processes.

Another significant point that should be considered is that in this study sampling was done from the honeybees inside the hive therefore, collected individuals represent adult workers from all classes of division of labor in the colony. However, significant variations at *Pgm* locus were observed among the honey bee workers that perform a variety of activities depending on their expenditure of energy which is associated with their work in the hive (unpublished data). In experiments performed in spring, the frequency of heterozygotes (*Pgm-75/100*) and homozygotes (*Pgm-75/75*) was almost equal in honey bees collected from the inside of the hive. This observation is in complete accordance with the present study, since in all locations studied genotype frequencies at *Pgm* locus followed a similar pattern in spring months. What is interesting was that the frequency of the heterozygote individuals was found to be significantly high among foragers compared to the bees collected from the hive ($P < 0.0166$). In addition, the frequency of *Pgm* heterozygotes among soldier bees was lower compared to the forager bees and higher as compared to the bees collected from the inside of the hive. Repeating the same experiment in different colonies in spring and summer months did not change the observed results and in all trials forager bees were determined to be significantly different from both the bees in the hive and the soldiers in terms of *Pgm* heterozygosity ($P< 0.0001$). In summer, as the results of this present study suggest the frequency of the *Pgm* homozygotes was significantly higher than heterozygotes, but at the same time foragers that were collected in summer were significantly different from summer bees collected from the inside of the hive. Therefore, we determined that the frequency of the *Pgm* heterozygotes among forager bees were significantly higher than the other workers in the hive. Since foragers and soldiers are of the same age, the observed significant difference in the frequency of *Pgm* heterozygosity is related with the work that they perform in the hive and the energy requirements of the activities they perform.

These observations together with the results of the present study indicate that energy requirement of the tasks performed by worker bees is significantly associated with *Pgm* genotypes which have functional and physiological consequences on the efficiency of the utilization of energy reserves, that is glycogen for honeybees. Individuals that are *Pgm* heterozygotes may have the advantage of increased efficiency in metabolic flux due to the increased PGM activity which in turn regulates the mobilization of glycogen reserves significantly. Since glycogen reserves are of primary importance during winter (thermoregulation) and summer (foraging), honeybees demonstrate a seasonal pattern of allele frequency changes at *Pgm* locus, however this seasonal pattern in allozyme frequencies is not independent of the socially regulated division of labor.

4.3. Conclusion

Monthly analysis of *Pgm* and *Hk* polymorphisms in three different locations have revealed that *Pgm* allele and genotype frequencies cycle through seasons in a pattern that the frequency of the heterozygotes (*Pgm-75/100*) increases during winter and drops suddenly in spring stays low through summer in three subspecies of honeybees each from different regions studied. PGM enzyme activity and glycogen content of the heterozygote individuals whose frequency increases in winter was determined to be significantly higher than those of homozygotes. Moreover, there is a significant positive correlation between enzyme activity and glycogen content of the honeybees.

More than one of the possible hypothesis stated throughout the text may combine to describe this interesting phenomenon in honeybees. Understanding the genetic, biochemical and behavioral mechanisms that lead to the observed seasonal variation in *Pgm* polymorphisms and its consequent association with energy metabolism requires further studies. Analysis of the gene expression profiles at *Pgm* locus in summer and winter bees can provide important information for the identification of molecular mechanisms that are responsible for the seasonal shifts in *Pgm* allozymes.

Seasonal changes in temperature may influence the expression of either the *Pgm* locus directly or indirectly through a regulatory locus that determine the allelic composition of the *Pgm* genotypes.

Honeybees are social insects and also social structure of a colony highly responsive to both the changes in environmental and within colony conditions (Huang and Robinson, 1992). Communication among the hive members by the use of pheromones has important physiological and behavioral consequences as Le Conte *et al*., (2000) have reported that certain pheromones can participate in the regulation of division of labor among adult worker bees and influence feeding behavior of honeybees (Le Conte *et al*., 1995). In addition, queen mandibular pheromone has been found to affect expression patterns of the genes in honeybee brain that are associated with nursing and foraging behavior (Grozinger *et al*., 2003). Therefore, we can not exclude the possibility that social interactions through the activity of the pheromones among bees can be a mechanism that may lead to changes in the expression pattern of *Pgm* alleles. Analysis of the *Pgm* genotypes of bees that belong to different classes of division of labor has demonstrated that among foragers *Pgm* heterozygotes were significantly in higher levels compared to other workers even in summer months in which heterozygosity is low in the hive (unpublished data). Therefore, the effect of social interactions can also be a subject of future analysis for the determination of the mechanisms that are responsible for the seasonal variation in *Pgm* allozymes.

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APPENDIX A

A.1. Reagents used in the Electrophoretic Analyses

	Name of the Reagent	Brand Name / Catalog Number
1	Starch	Sigma-S4501
2	Agar	Sigma-A7002
3	Glucose-1-Phosphate	Sigma-G1259
4	Glucose-6-PhosphateDehydrogenase	Sigma-G7877
5	$MgCl_2$	Sigma-M0250
6	NADP	Sigma-N0505
7	PMS	Sigma-P9625
8	MTT	Sigma-M2128
9	Tris	Sigma-T1378
10	ATP	Sigma-A2383
11	Glucose	Sigma-G8270

A.2. Reagents used in Enzyme Activity Assays

	Name of the Reagent	Brand Name / Catalog Number
1	Glucose-1-Phosphate	Sigma-G1259
2	Glucose-6-PhosphateDehydrogenase	Sigma-G7877
3	$MgCl_2$	Sigma-M0250
4	NADP	Sigma-N0505
5	Tris	Sigma-T1378
6	HCl	Sigma-H2435

A.3. Reagents used in Glycogen and Protein Measurements

	Name of the Reagent	Brand Name / Catalog Number
1	Glucose oxidase-peroxidase	Sigma-G-3660
2	o- dianisidine dihydrochloride	Sigma-D2679
3	amyloglucosidase	Sigma- 10115-5G-F
4	BioRad Protein Assay Dye Reagent	BioRad -500-0006
5	Bovine Serum Albumin Standard	Sigma-A2153
6	HCl	Sigma-H2435

APPENDIX B

B.1.Equiment used in the study

	Name of the Reagent	Brand Name / Catalog Number
1	Multipor II Electrophoresis Unit	Amersham
2	Power Supply	Amersham/ EPS 3501
3	Cooling Device	Heto
4	UV/Visible Spectrophotometer	Shimadzu
5	Microplate Reader	BioRad/ 680
6	Centrifuge	Eppendorf/5415R
7	pH meter	Eutech/Cyberscan 500

APPENDIX C

C.1. List of Symbols

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-TetrazoliumBromide

C.2. List of Mathematical Formulations

2.1. Enzymatic catalysis performed by PGM
2.2. Enzymatic catalysis performed by HK
2.3 Mathematical Formulation of the Beer-Lambert Law
2.4. Mathematical Formulation of the frequency of an allele in a population
2.5. Mathematical formulation of the observed heterozygosity
2.6. Mathematical formulation of the calculation of glycogen and protein concentration
2.7. Equation for the determination of the glycogen and protein concentration
2.8. Two step illustration of the PGM activity reaction
2.9. Mathematical formulation of the equation for the calculation of the PGM enzyme activity