DETERMINATION OF POLYMORPHISM OF PGM, HK, PGI, AND G6PD IN DIFFERENT DEVELOPMENTAL STAGES OF HONEY BEE (APIS MELLIFERA L.) AND ITS RELATION WITH PGM ACTIVITY AND GLYCOGEN CONTENT

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ABSTRACT

DETERMINATION OF POLYMORPHISM OF PGM, HK, PGI, AND G6PD IN DIFFERENT DEVELOPMENTAL STAGES OF HONEY BEE (*Apis mellifera* L.) AND ITS RELATION WITH PGM ACTIVITY AND GLYCOGEN CONTENT

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In this study, three subspecies of *Apis mellifera* L. (*A. m. caucasica, A. m. carnica, and A. m. syriaca*) from different climatic regions were evaluated electrophoretically at ontogenetic level by means of four enzymes, namely Phosphoglucomutase (PGM), Hexokinase (HK), Phosphoglucose isomerase (PGI) and Glukose-6-phosphate dehydrogenase (G6PD). It is determined that only *Pgm* and *Hk* loci were polymorphic. Allele and genotype frequencies at *Pgm* locus changes seasonally whereas *Hk* locus does not exhibit seasonal variation. Within the scope of this study we investigated at which developmental stage shifting to heterozygotes prior to winter occurs.

It is found that there is a seasonal fluctuation throughout the year in *Pgm* genotype frequencies at each developmental stage studied and correlated with enzyme activity and glycogen content. As the studied enzymes have crucial

roles in insect energy metabolism, results of this study provided further information about the relationship between carbohydrate metabolism and enzyme polymorphism of honey bees.

Keywords: *Apis mellifera*, population genetics, ontogeny, enzyme polymorphism.

ÖZ

BAL ARISINDAKİ (*Apis mellifera* L.) FARKLI GELİŞİM EVRELERİNDE PGM, HK, PGİ VE G6PD ENZİM POLİMORFİZMİNİN BELİRLENMESİ VE BUNUN PGM AKTİVİTESİ VE GLİKOJEN MİKTARIYLA İLİŞKİSİ

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Bu çalışmada, *Apis mellifera* L.'nın farklı iklimsel bölgelerden üç alttürü (*A. m. caucasica, A. m. carnica and A. m. syriaca*) ontogenetik seviyede dört enzim kullanılarak [Fosfoglukomutaz (PGM), Hekzokinaz (HA), Fosfoglukoz isomeraz (PGI) and Glukoz-6-fosfat dehidrogenaz (G6PD)] elektroforetik açıdan değerlendirilmiştir. *Pgm* ve *Hk* lokuslarının polimorfik olduğu belirlenmiştir. *Pgm* lokusundaki alel ve genotip frekansları mevsimsel olarak değişirken *Hk* lokusu mevsimsel varyasyon sergilememiştir. Bu çalışma kapsamında, kış öncesinde heterozigotlara geçişin hangi gelişim evresinde gerçekleştiği incelenmiştir.

Çalışılan her gelişim evresinde, *Pgm* genotip frekanslarında yıl boyunca enzim aktivitesi ve glikojen miktarıyla da ilintili mevsimsel bir dalgalanma olduğu tespit edilmiştir. Çalışılan enzimlerin böceklerin enerji metabolizmasında

oldukça önemli rol oynamaları dolayısıyla, bu çalışmanın sonuçları bal arılarının karbonhidrat metabolizması ve enzim polimorfizmi arasındaki ilişki hakkında aydınlatıcı bilgiler sunmaktadır.

Anahtar sözcükler: *Apis mellifera*, populasyon genetiği, ontogeni, enzim polimorfizmi.

To my family

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CHAPTER 1

INTRODUCTION

1.1. Taxonomy and Evolution of *Apis mellifera* L.

Apis mellifera L. (1758), also known as western honey bee, was first described by Linnaeus. The genus *Apis* is classified into eleven species and three subgenera based on morphological, ecological and molecular data (Engel, 1999; Takahashi, 2006). Among these species, 10 species are recognized and the validity of one species (*A. laboriosa*) is still not obvious (Arias and Sheppard, 2005). Morphological and molecular data are in agreement on the three subgenera: subgenus *Micrapis* (*A. andreniformis* and *A. florea*), subgenus *Megapis* (*A. dorsata* and *A. laboriosa*) and subgenus *Apis* (*A. mellifera, A. koschevnikovi, A. cerana, A. nigrocincta and A. nuluensis*) (Takahashi, 2006).

Honey bees are classified under the *Apinae* subfamily of *Apidae* family. The subfamily of *Apinae* distinguishes from other Apidae members by a few morphological and behavioural criteria such as: stretching of the pattern of wing venation, clustering behavior, progressive feeding of larvae, and communication by "dance language" (Ruttner, 1988).

According to morphometric (Ruttner, 1988), DNA sequence data (Cornuet and Garnery, 1991; Arias and Sheppard, 1996), and SNP analysis (Withfield et al., 2006) the speciation of *A. mellifera* L. occurred in Africa.. Whitfield *et al*. (2006) reported that *A. mellifera* expanded into Eurasia at least twice and these

expansions lead to geographically close but genetically distant populations of honey bees in eastern and western Europe.

Genus *Apis* contains the true honey bees which are highly eusocial insects. Characteristics specific to genus *Apis* first emerged in early Tertiary, and in the early Pleistocene an *Apis* type which had adapted to temperate climate evolved with new behavioral properties such as cavity nesting, temperature homeostasis, and a more complicated dance language (Ruttner, 1988). Homeostasis of temperature is maintained by clustering behavior which helps to regulate microclimate in hive and adaptation to temperate climate (Ruttner, 1988).

The taxonomic status of the *A. mellifera* L. is:

Kingdom: Animalia
Phylum: Arthropoda
Class: Insecta
Order: Hymenoptera
Suborder: Apocrita
Family: Apidae
Subfamily: *Apinae*
Genus: *Apis*
Species: *Apis mellifera*

The western honey bee, *A. mellifera* L., naturally distributed in Africa, Europe, and western Asia (Ruttner, 1988; Frank *et al.*, 1998). Current habitats of this species spread across the world due to multiple migrations and introductions (Ruttner 1988). Due to variety of geographical, climatological and ecological conditions, 26 subspecies are described based primarily on morphological characters in this vast area (Ruttner, 1988; Sheppard *et al*., 1997; Engel, 1999). These subspecies consist of four distinct evolutionary branches: African (A

lineage), Western and Central Asian (O lineage), Eastern European (C lineage), and Western and Northern European (M lineage) (Ruttner, 1988; Winston, 1997; Whitfield *et al*., 2006). The subspecies in Turkey belong to the Mediterranean C and O lineages and African A lineage (Kandemir *et a*l., 2006; Özdil *et al*., 2009).

1.2. Geographical Distribution of Studied Subspecies of *A. mellifera*

A. mellifera has adapted to many kinds of climate ranging from cold temperate to tropical, from stable humidity to semi-desert conditions and so different brood patterns, different rates of reproduction ranging from long-living colonies with low reproductivity to short-living colonies with a higher reproductivity evolved in this species (Ruttner, 1988). Because of this wide geographical distribution throughout the world, highly divergent subspecies evolved within this species.

Different subspecies appeared throughout the geological time due to the fact that Turkey has diverse climatologic and geographical conditions and different habitats. There are five honey bee subspecies in Turkey: *A. m. carnica* (Pollman, 1879*)* in Thrace, *A. m. caucasica* (Gorbachev, 1916) in the northeastern, *A. m. meda* in eastern, *A. m. syriaca* (Buttel-Reepen, 1906) in southern and *A. m. anatoliaca* in the rest of Anatolia (Ruttner 1992; Kandemir, 2000).

1.2.1. *A. m. carnica*

The scientific name of this subspecies derives from the name of the region Carnia in the South of the Austrian-Italian border (Ruttner, 1988). Known as “Carniolan Bee” this honey bee is easily described with dark body color and short cover hair. *A. m. carnica* is mainly distributed in northern and eastern Europe. This subspecies is adapted to continental type of climate with long,

cold winters and quickly changing into hot, dry summers (Ruttner, 1988). They are able to overwinter in smaller numbers of winter bees and during summer *A. m. carnica* tends to increase brood production (Ruttner, 1988). But they tend to swarm when they are overcrowded. When compared to other subspecies, carnica tends to drift less from one hive to neighboring hives. As far as their defensive behaviour is concerned, *A. m. carnica* is one of the most gentle subspecies.

1.2.2. *A. m. caucasica*

Beekeeping has begun in Anatolia before 1300 B.C. (Ruttner 1998). Also known as "grey Caucasian mountain bee", *A. m. caucasica* is a well known subspecies all over the world and has been used in beekeeping for more than one hundred years (Pollmann 1889). This subspecies has the longest proboscis of all subspecies of *A. mellifera* (Ruttner,1988). This subspecies is naturally distributed in east coast of the Black Sea, Georgia, northeastern Anatolia and parts of Azerbaijan (Alpatov, 1948; Bilash *et al*., 1976; Avetisyan 1978). So, this subspecies seems to be limited by a humid-subtropical climate at the sea level and cool temperate climate at mountainous zone (Ruttner 1998). Characteristics of *A. m. caucasica* can be summarized as being very gentle, having low swarming tendency, excessive use of propolis and poor winter survival probably due to *Nosema* sensitivity (Alpatov, 1932; Avetisjan, 1978; Ruttner, 1988).

1.2.3. *A. m. syriaca*

Known as "Syrian honeybee", this honey bee is the smallest of all oriental and European subspecies of *A. mellifera* (Bodenheimer, 1941). *A. m. syriaca* is naturally distributed around East Mediterranean region including Israel, Jordan, Lebanon, Syria, and Hatay region of Turkey (Ruttner, 1988). As reported by Adam (1983), these bees are very sensitive to cold climate but they are well

adapted to their native ecological conditions and they are good nectar collectors and show aggressive behaviour (Ruttner, 1988). According to morphometric analyses, this subspecies is the closest subspecies of the Middle East region to African honey bees (Ruttner 1988).

1.3. Biology of Honey Bees

Honey bee colonies comprise zero to few thousand drones, a single queen and usually 20-40 thousand of her worker daughters (Page and Peng, 2001). Because of haplo-diploid genetic system, the queen determines the sex of her offspring within the order Hymenoptera. Fertilized eggs produce diploid female offspring (workers and queens), whereas (except in *A. m. capensis* workers) unfertilized eggs develop as haploid males (drones). The fact that in terms of genetic basis workers are more closely related to the brood they rear than to their prospective offspring is believed to be the result of existence of altruistic behaviour and evolution of eusociality in this group (Hamilton, 1964). Despite having some analogies, eusociality evolved independently in *Meliponinae* and *Apinae* subfamilies (Winston and Michener, 1977). Often three characteristics are used to define eusociality: overlapping generations, cooperative brood care, and division into reproductive and sterile castes (West *et al*., 2007). Eusocial animal societies like ants, wasps, bees are structured around one or few reproductive females and nonreproductive females are called ‘workers’ which perform tasks including foraging, nursing, hoarding in the context of division of labor and this age-related divison of labor is belived to be crucial for fitness in terms of the individual performing the behaviour, and for the others in the population (West *et al*., 2007; Amdam and Page, 2010). Since workers are nonreproductive individuals, their labor could be shaped by natural selection in order to increase colony fitness (West- Eberhard, 1996).

One of the characters specific to *Apis* genus is the behavior of winter clustering which is the major factor of protecting the nest and regulating its microclimate (Ruttner, 1988). Thus, this behavior provides adaptation to temperate climate. Also as another factor to adapt to variable environmental conditions, the special system of communication in the genus *Apis* provides the chance to reach food sources around 6 to even 10 km around the nest. (Ruttner, 1988).

Honey bees are important elements in sustainable agriculture and ecosystem because while collecting pollen and nectar in order to maintain their survival, they contribute to the pollination of plants. According to FAO statistics, about 80 % of agriculturally important crops are pollinated by animal pollinators most of which are bees (FAO, 1980).

1.3.1. Ontogenetic Development of Honey Bees

The term ontogeny describes the developmental history of an organism from the fertilized egg to its mature form. Honey bee became an important model organism for investigation of developmental biology, senescence (Amdam and Omholt, 2002), reverse genetics, social behavior (Robinson et al., 2005) and brain biology (Heisenberg, 2004).

1.3.1.1. Larval Stage

Each larva develops in an individual open cell. During ontogenetic progression, young adults (nurse bees) produce a fluid substance called jelly and feed larvae until they develop into pupae. The ingredients of this larval food is mainly produced from polen which is the protein source of honey bees (Moritz and Crailsheim, 1987).

Larval development is completed within 5-6 days but 2-3 days of differential feeding causes profound differences in development (Page and Peng, 2001). As

queens are genetically identical to workers, differential feeding of female larvae with different amounts of royal jelly determines the developmental destiny of the honey bee embryo (Drapeau *et al*., 2006). It was reported that queen-destined larvae receive more larval food which is richer in certain sugars whereas worker-destined larvae are fed with a less rich diet (de Wilde and Beetsma, 1982).

Although there is a generalized time interval for development of larvae, bees may respond to changing social conditions by accelerating, delaying, or reversing their typical pattern of behavioral maturation (Remolina *et al*., 2007). It was observed that, when there is a shortage of foragers or large numbers of young larvae are available in the hive, some bees delay their transition to foraging and continue to serve as nurses (Robinson *et al*., 1989).

1.3.1.2. Pupal Stage

When larvae develop into pupae, nurse bees isolate pupae until eclosion by capping the comb with wax. Pupal development of queens and workers differ substantially in duration (Groh and Rössler, 2008). Development of queens are faster at pupal stage when compared to workers (6–7 days in queens compared to approximately 11 days in workers at this stage) (Groh *et al*., 2006). As Groh and Rössler (2008) summarized, in worker caste the postcapping period (the time after brood-cell sealing) is approximately 11 days, two prepupal and 9 pupal stages (P1–P9) with each stage lasting 1 day whereas in queen cast postembryonic development included one prepupal (1 day) and six pupal stages (6 days).

The amount of brood (larvae and pupae) and the efficiency of brood rearing is primarily determined by the amount of pollen in the hive which is influenced by weather conditions, so that 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).

1.3.1.3. Adult Stage

Following the 21 days of egg, larval and pupal stages, young workers emerge as winged adults. Adult stage of honeybee colonies comprise three casts: a single mated queen, her worker daughters and drones. Cast determination is independent of genetic basis because queen is the only reproductive female so all the individuals in the colony are her offspring.

Life time of the queen is longer than workers. Queens generally live less than a year in commercial hives, however it was reported that queens may live 2-3 years but this is not a usual situation (Page and Peng, 2001). The queen usually mates only once in her life with 12-15 drones and stores the sperms in her abdomen. Seasonal differences influences egg laying behavior: in winter very few eggs are produced whereas in summer 2000 eggs are laid up per day (Bodenheimer, 1937). Workers understand the presence of a queen by pheromones. As long as queen and her brood produce pheromones, workers do not become fertile and lay eggs (Barron *et al*., 2001). In addition to this situation, new queens are produced prior to swarming. When the virgin daughter queens are ready to emerge, the motherqueen and approximately half the workers leave the nest to establish a new colony. At that time, the first emerged queen kills her competitors when they are still at pupal stage or when they newly emerged.

Fertilized eggs become workers in the colony that perform all the tasks required for the survival of the colony. Unfertilized eggs become drones and their main task is to fertilize the queen.

Younger workers perform tasks inside the nest, such as feeding larvae (nursing), constructing and maintaining the nest and processing honey, while older bees go out of the hive to collect food (foraging) and defend the nest. Foraging task is further divided into specialists like polen (protein source) collectors and nectar (carbohydrate source) collectors (Amdam and Page, 2010).

Workers lifetime is generally 3-6 weeks during spring and summer whereas they can live up to 4 months during winter (Page and Peng, 2001). In honeybee societies, adult workers spend the first 2-3 week of adult life working in the hive and for the remaining 1-3 weeks they mostly serve as foragers (Robinson 1992). This age-related division of labor among workers is often associated with changes in physiology and morphology (Amdam and Page, 2010).

1.4. Seasonal and Temporal Changes in Honey Bees

Because body temperature of ectotherms is highly affected by temperature, surrounding temperature is major determining factor of geographic distiribution and abundance of ectotherms (Bowler and Terblanche, 2008). Members of the genus *Apis* are distributed throughout the world in highly diverse climates and its ecotypes have adapted well to their biotopes (Le Conte and Navajas, 2008).

Due to seasonal changes honeybee colonies exhibit various differences in terms of behavior, physiology, population structure and age demography (Bodenheimer, 1937; Merz *et al.*, 1979). While workers perform foraging brood rearing tasks actively with high numbers of individuals in summer, situation turns in to a hybernating broodless colony with small number of bees in winter (Matilla and Otis, 2007). Investigation concerning this transition demonstrated that possible reasons can be photoperiod, availability of pollen sources and temperature. (Matilla and Otis, 2007).

During winter all workers, which form winter clusters, produce heat by constriction of their thorasic muscles to maintain high temperature inside the nest (thermoregulation) (Esch, 1964; Crabtree and Newsholme, 1975; Beenakkers *et al*., 1984). Maintaining high temperature is achieved by breakdown of the carbohydrate sources (Stabentheiner *et al*., 2002). This adaptation to northern temperate climate is associated with social food-hoarding behaviour (Heinrich, 1994). As honey bees do not perform foraging tasks during winter, without the behavioural adaptation of food-hording the colony can not survive. Winter clusters are formed when surrounding temperature decreases to 15 ^{0}C and consist of two parts: inner core of actively contributing to heating bees and outher shell relatively lethargic bees (insulating part).

In addition to thermoregulation, another reason of how honey bee colonies survive in winter in spite of very little brood reared is longer life span of winter bees reaching an average of 140 days (Fukuda and Sekiguchi, 1966), while adult summer bees have an average life span of 15-38 days (Ribbands, 1952; Fukuda and Sekiguchi, 1966; Michener, 1974).

Le Conte and Navajas (2008) investigated the possible impacts of climate change on honey bees and concluded that these impacts may be seen at different levels: direct influences on behaviour and physiology, change in harvesting capacity of the honeybee colonies because of changing floral environment and differences in distribution ranges.

In previous studies Hadımoğulları *et al*. (2000) reported that there is a high level of heterozygosity at *Pgm* locus and heterozygositiy levels change in line with the seasonal conditions in Hatay regjon where *A. m. syriaca* exists. After this interesting phenomena noticed, Kence and Güldüren (2008) extended the investigation to three subspecies, *A. m. carnica, A. m. caucasica and A. m. syriaca*, with a long-term analysis and demonstrated that genotype frequencies

at *Pgm* locus fluctuate due to seasonal changes in these three subspecies and also, this seasonal fluctuation of enzyme polymorphism is associated with PGM activity and glycogen content levels of individual bees.

1.5. Allozymes as Genetic Markers

Molecular markers, which are closely related to advances in molecular biology, are widely used in biology to account for ecological, genetic and evolutionary relationships at the inter and intra specific levels (Arias *et al*., 2006). Protein markers have been used widely and made a significant contribution to studies concerning genetic variation within and between populations.

Allozyme electrophoresis is a useful biochemical technique that is used to detect electrophoretic patterns of allozymes in polyacrylamide or starch gels. Based on banding patterns of allozymes, heterogeneity level of a locus is determined in a population (Behura, 2006). Isozymes are also be used for the same purpose. The term allozyme refers to different allelic forms of a given enzyme coding gene, whereas the term isozyme refers to different forms of functionally similar enzymes encoded by different loci (Prakash *et al*., 1969; Buth and Murphy, 1998).

Allozymes, as molecular markers, enable us to make inferences about genetic structure of a population via allele and genotype frequencies, heterogeneity level, gene diversity, gene flow and enzyme polymorphism at a locus. With the help of these parameters, the situation of a population can be identified in terms of Hardy-Weinberg equilibrium and linkage disequilibrium and the results can be evaluated according to deviations from the equilibrium.

Allelic composition observed on the gel depends primarily on genotype of the organism, structure of the enzyme and the enzyme loci studied. Organism may

be haploid, diploid or polyploid in terms of nuclear condition and homozygous or heterozygous genetically (Micales and Bonde, 1995). On the other hand, allelic composition obtained from allozyme electrophoresis may be misleading about inferences of gene coding the enzyme because of transcriptional or post-translational modifications such as phosphorylation, deamination and acetylation. Also, variations in photoperiod and diet of the organism, sample processing procedures, sample storage time, and experimental methodology may also influence expression pattern of an enzyme and cause different allelic compositions on the gel (Poly, 1997). In order to avoid misinterpretations, standardization of sampling procedure and experimental methodology becomes crucial.

Because protein samples are more susceptible to laboratory conditions than DNA and greater level of polymorphism can be observed via DNA markers (Richardson *et al*., 1986), DNA markers became more common in studies aiming to measure genetic differences within and between species or populations (Behura, 2006). Although the choice of genetic marker depends upon the objectives of a study, there are inherenet advantages and disadvantages of each marker system which should be considered carefully (Behura, 2006).

1.6. Adaptive Consequences of Enzyme Polymorphism

After the 'neutral theory of molecular evolution' proposed at late 1960s (Kimura, 1968, Clarke 1970), 'neutralist-selectionist controversy' arose in the arguments concerning whether there are adaptive consequences of enzyme polymorhisms or not. The neutral theory claims that majority of evolutionary changes at molecular level are caused by random fixation of selectively neutral or nearly neutral mutants, not by Darwinian selection and most of the protein and DNA polymorphisms are selectively neutral (Kimura, 1986). This theory does not completely denies the role of natural selection in terms of adaptive

evolution but claims that only a very small fraction of changes in DNA are adaptive (Kimura, 1986).

Another theory called 'theory of metabolic flux' proposed supporting the assertion of selective neutrality, which was denoted in the neutral theory. According to this theory, variations in a single enzyme in terms of biochemical properties have not the ability to effect metabolic pathways because these pathways are under control of many loci which interact with each other through complex regulatory networks (Kacser and Burns, 1981). This means that allozymes or isozymes have no selective significance. Another inference of this theory is that *in vivo* conditions can not be completely imitated by *in vitro* conditions of experiments so biochemical differences observed via *in vitro* conditions can not reflect the real situation of an organism. However, there are contradictory examples demonstrating that *in vivo* measurements of enzyme activity can be consistent with *in vitro* measurements. For example, *in vivo* and *in vitro* activity measurements of 6-phosphogluconate dehydrogenase (6-PGD) and glucose-6-phosphate dehydrogenase (G6PD) allozymes exhibit consistency and different genotypes have different carbon flux rates through pentose shunt (Cavener and Clegg, 1981).

Under the assumption of influence of variation in just one enzyme on complex enzyme networks are undetectable (Kacser and Burns, 1981), it was claimed that selection must be acting on reproductive fitness of organisms, not on an individual enzyme loci, via changing the physiological output of variation at the overall metabolic reactions (Middleton and Kacser, 1983). But some researchers opposed to this evaluation by the idea that some enzymes functioning at regulatory points of metabolic pathways are more exposed to selection (Watt, 1985) and these enzymes in a metabolic pathway, may change the overall affect of metabolic flux and therefore change the fitness of an organism (Johnson, 1973). In addition to this opposition, recent studies dealing with the correlation between catalytic properties of allozymes and amino acid

sequences demonstrated that enzyme polymorphisms at branching points of pathways may be more exposed to selection pressures and have more potential to influence the metabolic flux (Eanes, 1999; Verrelli and Eanes, 2001). On the other hand, as electrophoretic techniques developed and became diverse, number of studies supporting the idea that genetic variation in natural populations is high and this variation causes fitness consequences. Moreover, it was proposed that electrophoretic methods are not able to detect total amount of genetic variation at an enzyme loci (Bernstein *et al*., 1973; Scozzari *et al*., 1979). Since the neutral theory first proposed many studies published both supporting the theory or contradicting this theory by suggesting that genetic variation may lead to significant fitness consequences in many organisms such as *Colias* butterflies (Watt *et al*., 1983, 1985) and *Drosophila melanogaster* (Verrelli and Eanes, 2001).

In a study investigating the consequences of amino acid polymorphism in *D. melanogaster* it was shown that different genotypes of *Pgm* locus has different catalytic capacity and there is a positive correlation between PGM activity and glycogen content (Verrelli and Eanes, 2001). Different studies conducted with *Alvinella pompejena* (polychaete worm) elucidated that *Pgm* has the highest polymorphism among all enzyme loci taking part in glycolysis and Krebs cycle (Jollivet *et al*., 1995) and different *Pgm* genotypes have different termostability profiles which influence geographic range of this organism (Piccino *et al*., 2004). In the environment of fluctuating temperature allelic variation at *Gpi* locus can increase the Darwinian fitness of sea anemone, *Metridium senile*, by modulating carbohydrate metabolism at glycolysis-pentose shunt branch with different kinetic properties of allozymes (Hoffman, 1981). reported that kinetic differences among allelic variants of *Gpi* in sea anemone, *M. 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. Also, it was reported by McMillan *et al*. (2005) that fitness of larvae and thermal behaviour

of adults are influenced by different physiological properties of different *Pgi* allozymes in leaf beetle, *Chrysomela aeneicollis*.

Seasonal variation may also affect the allozyme genotypes and cycling of allozyme polymorphism throughout the year can be a way of adaptation to fluctuating environmental conditions. According to the assumption of protein variation is temporarily constant, effects of seasonal changes on allozyme variation which has adaptive value were underestimated (Moffett and Crozier, 1996). As seasonal changes in allozyme frequencies occur as a response to temperature, Hochachka (1967) and Somero (2004) proposed that differential gene expression cause shifts in allozyme frequency according to fluctuating environmental conditions. Somero (2004) summarized the three possible mechanisms associated with enzymatic adaptation to temperature: first mechanism can be changes in primary structure (amino acid sequence) which cause changes in catalytic properties of an enzyme; secondly, changes in expression of the coding gene which changes the actual concentration of an enzyme; and lastly, changes in the molecular environment in which an enzyme functions.

These mechanisms were supported by various studies indicating that regulatory genes play a more important role via epigenetic effects than genetic variations at enzyme loci (Wilson, 1976; Ayala and McDonald, 1981; Allendorf *et al*., 1982) and inversions may lead to polymorphism at enzyme and chromosome level and in turn, these polymorphisms yield cycling genotype frequencies which occur in line with fluctuating seasonal conditions (Dobzhansky and Ayala, 1973).

Organisms response to environmental changes such as temperature, latitude, altitude etc. is due to morphological, behavioral plasticity and the genetic variation that provides physiological adaptations in a changing environment (Watt 1983, 1994; Wu, 1998; Dahlhoff and Rank, 2000; McMillan *et al*.,

2005). From evolutionary perspective, role of enzyme polymorphisms can be interpreted as a way of providing metabolic flexibility to adapt and respond the heterogeneous environments (Johnson, 1973).

1.7. Carbohydrate Metabolism of Honey Bees

There is a consensus on the idea that carbohydrates are the most important enegy source of honeybees (Panzenböck and Crailsheim, 1997). Carbohydrate sources of honeybees are comprised of nectar, honeydew and honey which is produced inside the colony whereas main protein source is polen (Panzenböck and Crailsheim, 1997).

Age polyethism is accompanied by physiological changes at the juvenile hormone levels, water content of the body, thorax glycogen content, metabolic rate, flight capacity and body mass (Harrison, 1986; Huang *et al*., 1994; Roberts and Elekonich, 2005). As an example, Roberts and Elekonich (2005) demonstrated that throughout the behavioural development of honeybees, activity levels of various enzymes change in different tissues. Furthermore, it has been reported that age-related division of labor response to social and physical environment such as seasonal changes, weather, nutritional conditions (Page *et al*., 1992; Giray and Robinson 1994; Huang and Robinson 1995; Giray *et al*., 1999).

Because of the age-dependent polyethism, feeding habits of honey bees are changing due to the task they perform. In turn, metabolic enzymes possessed by an organism may account for its dietary specializations (Kunieda *et a*l., 2006). For example, nursers consume more polen (Crailsheim *et al*., 1992) whereas foragers are in the need of more nectar or honey (Brandstetter et al., 1988; Heran and Crailsheim, 1988). Also, the amount of carbohydrates and especially glycogen is very important because it used as oxidative fuel for flight (Candy, 1989). Accordingly, glycogen content of honeybees increase

with aging from larval stage to the foraging bee and foragers have the highest glycogen content in flight muscles among all ages (Neukirch, 1982; Kunert and Crailsheim, 1988). Moreover, as foragers venture outside flight metabolic rate increases by 15% and this increase is associated with doubling of thoracic glycogen concentrations (Fewell and Harrison, 2002). In this sense, enzymes function in carbohydrate metabolism may have particularly more efficient roles during flight in the honeybees.

Panzenböck and Crailsheim (1997) reported that *A. m. carnica* have lower glycogen reserves in summer and in winter workers have different glycogen content that active bees from the core area of winter cluster has relatively lower glycogen levels when compared to inactive bees from the outher surface of the winter cluster. This variation may be due to increased metabolic rate of the bees in the core region to produce more heat and consume more food (Free, 1957; Panzenböck and Crailsheim, 1997). These authors also pointed out that as a possible adaptation mechanism to the risk of not being fed as intensely as young workers, drones have significantly larger amount of glycogen than young workers.

Insect flight is an aerobic process (Kammer and Heinrich, 1978) so oxygen consumption rates are used as a parameter of flight muscle capacity (Panzenböck and Crailsheim, 1997). Like flight, thermoregulation is an aerobic metabolic process in which per one mole of glucose consumed, 36 mol ATP and 2818 J heat are released (Southwick, 1991). Therefore, it can be confidently concluded that metabolic costs of thermoregulation and foraging tasks are very similar (Panzenböck and Crailsheim, 1997).

Flight task of insects has high metabolic costs because it requires the increased activity of several tissues (Candy *et al*., 1997). Insects has mass-specific oxygen consumption rates during flight among all animals (Sacktor, 1976), and this rate raises 100-fold during flight in contrast with the resting time (Rothe

and Nachtigall, 1989). Because insect flight muscle has very low activities of pentose phosphate shunt enzymes (Newsholme *et al.* 1972; Clark *et al.* 1973), more pyruvate is directed from glycolytic enzymes for mitochondrial oxidation (Staples and Suarez, 1997).

Increase in thoracic temperature and activity during flight may cause further increase in metabolic rates up to 50-100 fold (Kammer and Heinrich, 1978; Casey, 1998) via the increased activity of the certain glycolytic enzymes functioning at rates close to their maximal flux capacity (Suarez, 2000).

Forager bees carry nectar or pollen to hive and this load may reach to 80% of their body weight (Winston, 1987; Fewell and Harrison, 2002). Also, undertaker bees, which carry dead bees away from the hive, carry load approximately their total body weight during flight (Coelho, 1991). Consequently, these loads increases metabolic cost by 40 per cent and require high amounts of energy (Fewell and Harrison, 2002).

Several studies indicate that polymorphism at an enzyme locus has an influence on flight metabolism. Gu (1991) investigated the relationship between enzyme polymorphism and flight metabolism in terms of *δ-Gpdh*, *G6pd* and *Pgm* in light-brown apple moth, *Epiphyas postvittana*, and concluded that as the only polymorphic one among studied enzymes *Pgm* is correlated with the variation in the flight duration of the moths. Similar inferences were made in studies which detected correlations between insect flight metabolism and polymorphism in several enzyme loci which involve in cellular respiration and energy metabolism such as glycerol-3-phosphate dehydrogenase in *Drosophila* spp. (Barnes and Laurie-Ahlberg, 1986) phosphoglucose isomerase in *Colias* butterflies (Watt, 1983).

Among the genus *Apis, A. cerana* and *A. mellifera* bees fly with higher speed, have a higher thoracic temperature and so have a higher metabolic rate when compared to other *Apis* members (Ruttner, 1988). It is suggested that higher metabolic rate in these two Apis species is correlated with shorter life span of worker bees of these species which is in contrast to long-lived workers of other *Apis* members (Ruttner, 1988).

1.7.1. Studied Enzymes and Their Role in Energy Metabolism

Carbohydrate metabolism refers to biochemical processes responsible for the formation, breakdown and interconversion of carbohydrates (energy stores) in organisms (Klowden, 2002). Carbohydrates are a short-term fuel for organisms because they are easier to metabolize than fats and proteins. Accordingly, carbohydrates are the most important enegy source of honeybees (Panzenböck and Crailsheim, 1997). It is accepted that glucose is the most important carbohydrate is glucose, a monosaccharide metabolized by nearly all known organisms. As far as their specialized carbohydrate-rich diet and caste determination due to nutrient is considered, carbohydrate-metabolizing enzymes may have crucial roles in the honey bee. (Kunieda *et al*., 2006). Schematic representation of the basic reactions at the branch point of energy metabolism in honey bees is shown in Figure 1.

However coding genes of metabolic enzymes are one of the most conserved genes, dietary specialization of the bees on honey and pollen may have caused selective pressure on some metabolic pathways (Kunied *et al*., 2006). There are mainly six metabolic pathways concerning carbohydrate metabolism: carbon fixation, glycolysis, pentose phosphate pathway, glycogenesis, gluconeogenesis and glycogenolysis.

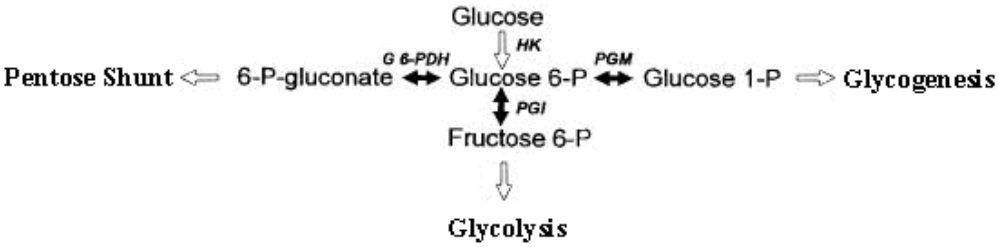


Figure 1. Schematic representation of the basic reactions at the branch point of the glycolytic pathway divided into the glycogen synthesis, the pentose shunt, and glycolytic corridor (from Dolgikh *et al*, 1997).

Glycolysis is a metabolic pathway that converts glucose into pyruvate and lactate and provides energy. Pyruvate enters the Krebs cycle for the aerobic oxidation to yield energy. Glycolysis is thought to be the archetype of a universal metabolic pathway because it is phylogenetically ancient and generally its features are strongly conserved (Hochachka and Somero, 2002). Hexokinase is the first enzyme functioning it this pathway. HK enables metabolisation of glucose via phosphorilating the 6 carbon on glucose. At this branch point, the phosphorilated glucose may continue through glycolysis or enter the glycogenesis to be converted into glycogen, or enter pentose phosphate pathway to be used in formation of amino acids and nucleic acids.

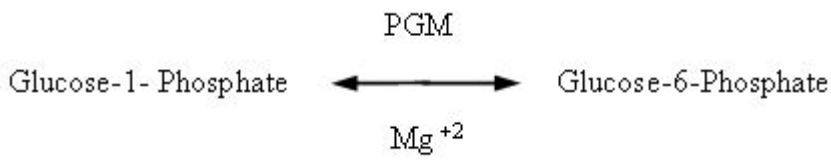
As an alternative pathway to glycolysis, the pentose phosphate pathway is responsible for the conversion of hexoses into pentoses and NADPH regeneration. While it does involve oxidation of glucose, its primary role is anabolic rather than catabolic. For most organisms, it takes place in the cytosol (Kruger and Schaewen, 2003). NADPH is reducing equivalent and used in biosynthesis reactions like fatty acid synthesis whereas other products of this pathway are used in formation of nucleic acids and aromatic amino acids. Glucose-6-phosphate dehydrogenase is the rate-controlling enzyme of this pathway.

Glycogenesis is the process responsible for the conversion of excess glucose into glycogen as a cellular short-term storage mechanism. This process is activated during rest periods in response to high glucose levels. Phosphogluco mutase plays a key role in this pathway by enabling Glucose-6-phosphate to enter this pathway. Glycogenolysis is the opposite process of glycogenesis which is responsible for the breakdown of glycogen into glucose.

Gluconeogenesis is the process enabling *de novo* synthesis of glucose molecules from simple organic compounds and results in the generation of glucose from non-carbohydrate carbon substrates. So, this process occurs during periods of such as starvation, low-carbohydrate diets, or intense physical activity. Pyruvate enters gluconeolytic pathway as substrate and glucose produced as the resulting product. Gluconeogenesis is a common and conserved process among plants, animals, fungi, and other microorganisms (Lehninger, 2000).

1.7.1.1. Phosphoglucomutase (PGM) E.C. 5.4.2.2

Phosphoglucomutase is a key enzyme in energy metabolism. It functions at a branch point of the glycolytic pathway dividing into the glycogen synthesis, the pentose shunt, and the main glycolytic corridor and catalyzes the reversible transfer of a phosphate group between glucose-1-phosphate and glucose-6-phosphate, the first intermediate in glycolysis. (Ray and Roscelli, 1964; Ray and Peck, 1972; Verrelli and Eanes, 2001).



After a single glucose molecule is broken off from the greater glycogen structure, the free glucose molecule has a phosphate group on its 1-carbon. This glucose-1-phosphate isomer cannot be metabolized easily. So, the enzyme

PGM phosphorylates the 6-carbon, while subsequently dephosphorylating the 1-carbon. The product of this reaction is glucose-6-phosphate, which can now theoretically travel down the glycolysis or pentose phosphate pathway.

The phosphorylated sugars may enter several different catabolic pathways, to yield energy (ATP) or enter anabolic pathways, leading to the synthesis of polysaccharides mainly glycogen in animal tissues (Ray and Roscelli, 1964). Therefore, PGM plays an important role in directing and regulating the metabolic flux of energy metabolism in all cells and functions in both glycolysis and gluconeogenesis (Ray and Peck, 1972).

PGM enzyme has a single subunit (monomeric in structure) and mainly found in cytoplasm of all cells.

1.7.1.2. Hexokinase (HK) E.C.2.7.1.1

Hexokinase catalyzes the first reaction in the glycolysis via the transfer of a phosphoryl group from ATP molecule to glucose to form Glucose-6-Phosphate. But in general this enzyme phosphorylates a six-carbon sugar, a hexose, to a hexose phosphate. In most tissues and organisms, glucose is the most important substrate of hexokinases, and glucose-6-phosphate the most important product. So, the role of HK is not as specific as PGM.

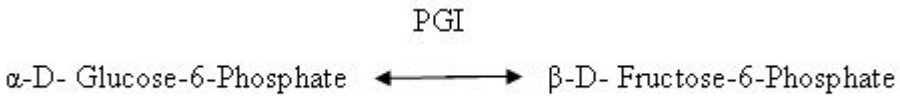
Phosphorylation of a hexose such as glucose often limits it to a number of intracellular metabolic processes, such as glycolysis or glycogen synthesis. Phosphorylation makes hexose unable to be transported out of the cell.

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

HK is one of the most widely distributed enzymes from bacteria to vertebrates, monomeric in structure and mainly found in the cytoplasm of most cells.

1.7.1.3. Phosphoglucose Isomerase (PGI) E.C.5.3.1.9

Phosphoglucose isomerase catalyzes the aldose to ketose isomerization of glucose-6-phosphate into fructose-6-phosphate in the second step of glycolysis (Staples and Suarez, 1997). Thus, it is highly conserved in bacteria and eukaryotes. This enzyme plays a central role in both the glycolysis and the gluconeogenesis pathways.



PGI is a widely distributed enzyme from bacteria to mammals, dimeric in structure and mainly found in cytoplasm of the cells.

1.7.1.4. Glucose-6-Phosphate Dehydrogenase (G6PD) E.C.1.1.1.49

Glucose-6-phosphate dehydrogenase is a cytosolic enzyme converting glucose-6-phosphate into 6-phosphoglucono-δ-lactone and is the rate-limiting enzyme of the pentose phosphate pathway, a metabolic pathway that supplies reducing energy by maintaining the level of the co-enzyme nicotinamide adenine dinucleotide phosphate (NADPH). This enzyme is stimulated by its substrate Glucose 6 Phosphate and increased utilization of NADPH will increase the level of $NADP^+$, thus stimulating G6PD to produce more NADPH.

G6PD is widely distributed in many species from bacteria to humans, monomeric in structure and mainly found in cytoplasm of cells.

1.8. Objective of the Study

Main objectives of this study are:

- To detect enzyme polymorphisms of PGM, PGI, HK, and G6PD at three developmental stages of three subspecies of *A. mellifera* those were adapted to different ecological and climatic conditions. As these enzymes play crucial roles in carbohydrate metabolism of insects, results of this study may provide further information about insect energy metabolism.

- To elucidate at which stage of development selection begins to act against homozygotes with the arrival of winter and if there is a seasonal fluctuation pattern in every stage.

- To investigate if there is a correlation between seasonal fluctuations of *Pgm* genotype frequencies and PGM enzyme activity, glycogen, and protein contents at different stages of ontogenetic development.

CHAPTER 2

MATERIALS AND METHODS

2.1. Biological Materials

2.1.1. Samples Used for Allozyme Analysis

Totally 2160 worker honeybees were studied in allozyme analysis of PGM, PGI, HK and G6PD enzymes (Table 1). Since drones are haploid and no heterozygosity can be observed, they are excluded. Also, for each of the larval and pupal stages and eggs, allozyme analyses were carried out with the samples at the middle of their developmental interval. Therefore, 1-3 day eggs, 3-4 day larvae, and 6-8 day pupae (at the onset of pigmentation) are used for all of the allozyme, protein, glycogen and enzyme activity assays. Samples were collected and brought alive to the laboratory. Larvae were washed with distilled water to remove royal jelly around them. Then they were labeled and kept at -80 until homogenization.

2.1.2. Samples Used for Enzyme Activity Assays, Glycogen and Protein Measurements

For each of the developmental stages (egg, larval, pupal and adult stages), all assays are conducted with 60 individual bees (20 individuals for each of the subspecies) collected in summer and winter months. Same samples were used in all enzymatic, protein and glycogen measurements. To avoid a decrease in enzyme activity, fresh samples were used in PGM activity assays. After these assays were performed, allozymic analyses were done with the same samples to detect their genotypic differences.

2.1.3. Chemicals Used in the Analyses

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

2.2. Methods

2.2.1. Sampling

Since the honey bee (*A. mellifera* L.) workers of individual colonies are descended from a single queen, instead of sampling few bees from several colonies, we preferred to make extensive sampling of a low number of colonies from a single location. By this way, a valid inference about the studied populations could be possible.

The hives which samples were collected for this study were kept at the common garden in the Middle East Technical University (METU) campus originally brought from three different geographical locations (Figure 2). To see the effects of climate and geography on populations, we sampled three subspecies from northeastern, northwestern and southeastern Anatolia. Original locations of sampled honeybees, sample collection intervals and number of bees studied per month and the total number of worker bees studied throughout the electrophoretic analysis is given in Table 1.

Table 1. Original locations of sampled honeybees, sample collection intervals and corresponding samples sizes.

Location	Name of subspecies	Sample Collection Interval	Sample Size / Month (Adult-Worker)
Northwestern Anatolia	*A. mellifera carnica*	July 2008-December 2009 (18 months)	40
Northeastern Anatolia	*A. mellifera caucasica*	July 2008-December 2009 (18 months)	40
Southern Anatolia	*A. mellifera syriaca*	July December 2009 (18 months)	40
TOTAL			2160

Figure 2. Geographic location of colonies which are taken to the METU campus.

Northwestern, northeastern and southeastern Anatolia exhibit different climatic and ecological conditions, extending from temperate to subarctic and subtropical climate.

A. m. carnica populations were brought from Kırklareli. This city is located in the Thrace region in which temperate climate prevails with four seasons, dry summers and moderate precipitation in the rest of the year. The natural vegetation is mainly composed of deciduous forest and bushy vegetation.

A. m. caucasica populations were brought from Artvin. This city is located in northeastern Turkey in which a kind of subarctic climate prevails with harsh winters and a long snow cover up to 8 months from October to early June. The natural vegetation is mainly composed of coniferous and deciduous forests. Artvin-Camili is a biosphere reserve announced by UNESCO at 2005 (World Network of Biosphere Reserves, 2010) and this area is under protection by Turkish Ministry of Environment and Forestry. Migratory beekeepers are not allowed to enter into this region.

A. m. syriaca populations were brought from Hatay. This city is located in southeastern Turkey in which subtropical Mediterranean climate prevails with warm temperatures throughout the year and high precipitation rates in winter. The natural vegetation is mainly composed of scrub and forests.

2.2.2. Electrophoretic Analysis

2.2.2.1. Preparation of the Homogenates

Thoraces of worker adults and pupae were dissected and homogenized in 300µl distilled water, as larvae were not dissected and homogenized as a whole in 250µl distilled water on ice to prevent any loss of enzyme activity. Then, homogenates centrifuged for 15 minutes at 12000 rpm and at +4°C. Supernatants are transferred in microtubes and stored in – 80 °C until used.

2.2.2.2. Electrophoresis

Electrophoresis is widely used in biochemistry for separation of biological molecules like amino acides, proteins, and nucleic acids. Because electrophoresis is a non-denaturating technique and highly sensitive to small sample size, it is one of the most useful research tools in the analysis and characterization of genetic variation in natural populations. This is a kind of separation technique based on the rates of movement of molecules when subjected to an electric field in a buffer system. Under these conditions, a charged molecule migrates in the direction of the electrode bearing the opposite charge. Because electrophoretic mobility depends on strength of electric field, temperature, concentration and the nature of gel material, charge of ion, size and shape of moving ion; different molecules migrates at different velocities and separate from each other.

In this study, thin layer native horizontal starch gel electrophoresis was used. The basic equipment for electrophoresis is illustrated schematically in Figure 3.

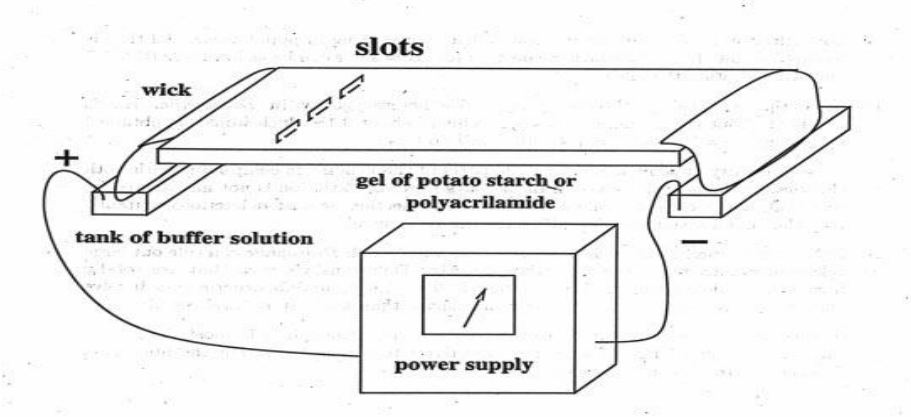


Figure 3. Illustration of the horizontal starch gel electrophoresis equipment.

2.2.2.3. Preparation of the Starch Gel

Quality of the prepared starch gel may be affected by some variables, such as brand of starch, the quality of distilled water, starch lot, laboratory and

refrigerator temperatures, and the length of time that gels are vacuum-degassed (Conkle *et al*., 1982). In preliminary studies different starch concentrations have been experimented and the concentration of 8-9% was found to be most suitable for the studied enzymes and this concentration is maintained throughout the study.

Procedure:

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

- 8.8 % (for PGM, HK, PGI) and 9% (for G6PD) starch gel solutions with a total volume of 300 ml (V_T= 270 ml distilled water and 30 ml buffer) were prepared in a flask. The gel swirled to make all the starch evenly suspended in the solution and cooked until the gel thickens on a bunsen burner.

- Then the gel was degassed with a vacuum pump for 15-20 seconds until small bubbles disappear and large bubbles (water vapor) appear.

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

- After a cooling period of 10-15 minutes, the gel mold was put in to the refrigerator to fasten polymerization. After 50-60 minutes, when the gel becomes opaque. The upper layer of the gel mold was sliced before loading the samples to get a flat surface.

2.2.2.4. Loading and Running Gels

Table 2. Electrophoretic conditions for the studied enzymes.

Enzyme	Buffer System	Running Temperature	Voltage & Current	Running Time	References
PGM	Tris-Maleic pH. 7.4	4 °C	170 V 30mA	4hrs	McDonald, 1985 Shaw&Prasad, 1970
HK	Tris-Citrate pH. 8.5	4 °C	150 V 15mA	5hrs	Shaw&Prasad, 1970
PGI	Tris-Citrate pH. 8.5	4 °C	250 V 25mA	5hrs	Shaw&Prasad, 1970
G6PD	Tris-Versene-Borate pH. 8.0	4 °C	175 V 8.6mA	6hrs	Shaw&Prasad, 1970

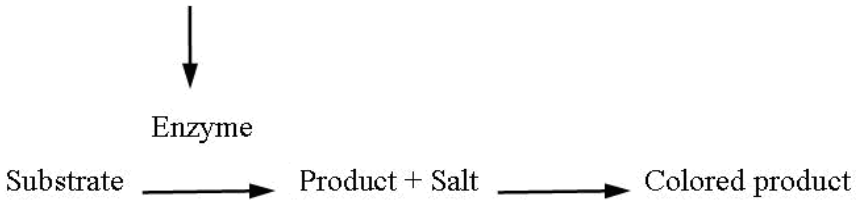
Loading process was performed on ice and all the homogenates were kept in ice to avoid any decrease in enzyme activity. After centrifugation of homogenates, supernatant was absorbed by Whatman no 3 filter paper and the filter paper was inserted into sample slots on the starch gel for electrophoretic analysis. Two paper wicks were dipped into Bromophenol Blue solution and loaded both sides of the sample slots as a marker in order to monitor electrophoresis process when the current is on. 250 ml of +4 ^{0}C electrode buffer (given in Table 2) was poured into electrode trays and the gel was placed on the cooling plate of the gel tank. The sponge wicks were saturated with buffer were placed on both ends of the gel and extended down into the tray to contact electrode buffer. Then the voltage and the current of the power

supply turned on. The cooling unit of the electrophoresis tank maintained at 4 °C throughout the run.

2.2.2.5. Staining

The most suitable electrophoretic system and running conditions are given for the studied enzymes (Phosphoglucomutase - PGM, Hexokinase - HK, Phosphoglucose isomerase - PGI, Glucose-6-phosphate dehydrogenase - G6PD) studied in Table 2.

After the application of the electric current to the gel for a sufficient time, gel is stained histochemically by soaking it in a solution containing the enzyme-specific substrate and cofactor, and oxidized salt along with a dye that precipitates where the enzyme catalyzed reaction occurs (Soltis *et al*., 1983). And, products of enzymatic reaction can be seen as distinct dark bands on the gel. This method is also called as "Activity Staining" because the success of the staining depends on the maintenance of the activity of the proteins throughout the electrophoresis. During staining process, the following reaction takes place:



In order to immobilize stain solution on gel slices, agar overlays were used during staining pocess (Whitmore, 1990). Then, the gels were kept in a dark incubator at 37°C. Since stains containing PMS and MTT turn blue after 15-20 minutes in the light, a dark incubator is essential. The staining ingredients for the studied enzymes were given in Table 3.

Table 3. Staining ingredients 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
PGI	Tris-HCl pH. 8.0 staining buffer Fructose-6-Phosphate $MgCl_2$ NADP Glucose-6-Phosphate Dehydrogenase PMS MTT Agar Solution
G6PD	Tris-HCl pH. 7.1 staining buffer Glucose-6-Phosphate NADP PMS MTT Agar Solution

2.2.2.6. Interpretation

Interpretation of the electromorphs on a gel was done according to primary and quaternary structure of the enzyme studied. If there is a variation in the primary structure (amino acid sequence) of a protein, net charge of this protein changes and it behaves differently under electric field and this is seen on the gel as genetic variation. Also, if an enzyme is monomeric (has a single polypeptide unit), homozygotes have a single band and heterozygotes have two bands (when there are two or more alleles in the population). And, every different band on the gel directly results from different polypeptide coded by a different allele. Accordingly, if an enzymes is dimeric (having two different polypeptide units) and this dimeric enzymes is coded by a single locus, then a heterozygote individual having two different alleles exhibits three bands on the gel because there are three possible combinations of the polypeptide subunits. And, every different band on the gel results from different combinations of polypeptide units.

Typical banding patterns of a monomeric and a dimeric enzyme coded by a single locus are illustrated in Figure 4. In this illustiration, sample 1,3,4,6 denote homozygote individuals whereas sample 2, 5 denote heterozygote individuals.

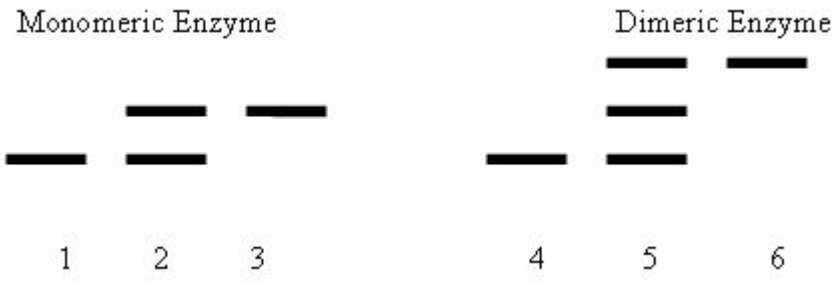


Figure 4. Banding patterns of monomeric and dimeric enzymes

2.2.3. Spectrophotometric Analysis

2.2.3.1. Basic Principles of Spectrophotometric Measurements

Spectrophotometric techniques are widely used in biological sciences to make quantitative and qualitative measurements. Because these techniques do not degrade the molecules analyzed and enable us to assay extremely small quantities of material (Lehninger, 2000).

Basically spectrophotometry is a technique that concentration of a solution is detected by measuring its absorbance of a specific wavelength via a spectrophotometer which produces light at a selected wavelength and direct it through a sample. Since every compound has a specific absorption spectrum, like a "fingerprint", we can identify and characterize its properties or detect its actual concentration in the presence of other compounds (Lehninger, 2000).

Two terms are used as indicators of the concentration of the solution in the sample (i.e.,cuvette). These are light transmission (refers to amount of emergent light after passing through a colorimeter tube or cuvette containing the light absorbing solution) and absorbance (refers to absorbance by the solution or depth of color of the solution). Absorbance is more frequently used in biochemical measurements. Because according to the Beer-Lambert Law, there is a linear relationship between concentration of absorbing solution and absorbance. This law mathematically states that absorbance of the solution is directly proportional to the concentration and the length of path through the solution (the width of the cuvette):

Beer-Lambert Law is applicable only at low cancentrations and so absorbance range should be 0 - 0.6.

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

A =Absorbance at a particular wavelength (no units)

ε = molar extinction coefficient for the absorbing material at a particular wavelength λ (M^{-1} cm^{-1}), 6200 M^{-1} cm^{-1} for NADH

C = molar concentration of the absorbing solution (M). A measure of how strongly a substance absorbs light at a particular wavelength.

d = length of light path through the solution (cm)

In the case of enzyme activity measurements, the assay solution contains some other compounds that are required for the reaction to occur. Other compounds in the reaction mix may absorb light at the same wavelength with the enzyme being analyzed. To eliminate the interference of other compounds, the absorbance of sample solution is compared with blank solution which is taken as the reference. The blank contains everything found in the sample solution except the substance to be assayed.

In the case of protein measurements, colorimetric techniques are used. Colorimetric analysis are performed via quantitative estimation of a coloured complex which is generally formed by the reaction of a colourless substance and a dye reagent. But the substance which will be analysed can be naturally coloured and can be read directly from the spectrophotometer.

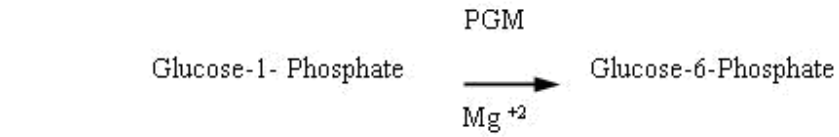
2.2.3.2. Determination of PGM Activity

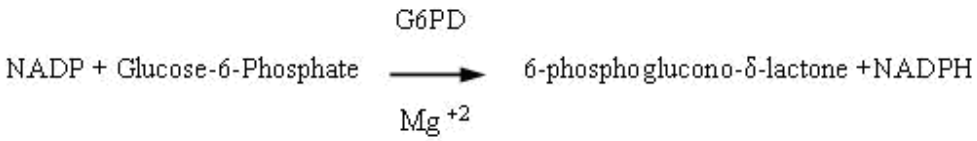
For each larval, pupal and adult stage of three subspecies, PGM activity assays were conducted with 20 individual bees which were collected in summer and winter months. Therefore, totally 180 individual bee were assayed in enzyme activity measurements. The individual bees used in enzyme activity assays were chosen from the freshest (latest collected) samples.

Frozen honeybees were dissected; thoraces of adult honeybees and pupa were homogenized in 300µl distilled water, and whole larvae were homogenized in 250 µl distilled water. Enzyme activity in honey bee eggs could not be detected because of insufficient amount of the enzyme. All the samples were centrifuged for 15 minutes at 12000 rpm to prevent the contamination of cellular debris and 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.

Enzyme activity of PGM was determined by measuring the increase in absorbance caused by the reduction of NADP at 340 nm at 25°C, pH 7.4. This enzyme assay is based on the conversion of $NADP^+$ to NADPH rather than a direct measure of PGM activity. Thus, this assay focuses on a secondary reaction, in a Glucose-6-phosphate dehydrogenase coupled system. These reactions are illustrated below:





In preliminary studies, firstly a variety of G6PD concentrations were assayed to understand whether there is a bottleneck in the second reaction of this coupled enzyme system and suitable G6PD concentration is determined for the reaction mix. According to this assay, PGM activity was calculated 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 Lambert Law was used to obtain concentration of NADH from ΔA_{340}/min values. To do this, we needed to define some volumetric measures used this equation such as:

- Total volume of assay (V_{total} = 0.6 ml)
- Volume of homogenate fraction used in the assay (V_{sample} = 0.033 ml)
- Dilution factor for each fraction analyzed
- Duration of reaction (t = 5 min)

Thus the equation for PGM activity is:

µmol of NADP reduced/min/ml =

$$\frac{\text{slope}}{6.2\ \mu M^{-1} cm^{-1}} \times \frac{0.6\text{ ml assay volume}}{0.033\text{ ml extract volume}} \times \frac{1}{d} \times \frac{1}{df}$$

Procedure:

PGM activity assays were optimized from the method used in Verrelli and Eanes (2001) and carried out on a Varian Cary 100 Bio UV-visible spectrophotometer equipped with a temperature-controlled cuvette holder.

The assay reaction mix was prepared immediately before performing the assay. The composition of the PGM activity assay mix is as follows:

0.85mM G1P

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

1.0mM $MgCl_2$

3.2 units/ml G6PD

The assay contained 567µl of the reaction mix and 33µl of homogenate (a total volume of 0.6 ml).

- The spectrophotometer instrument switched on for around 30 min before readings are made to allow the bulbs to warm up. The temperature of the spectrophotometer was set to 25ºC and maintained constant throughout the measurements.

- Parameters were entered as: stop time (5 minutes), cycle time (0.3 minutes), dwell time (10 sn), average time (1 sn) and wavelength (340 nm).

- A multicell and double beam option chosen in order to run 5 blank and 5 sample solution at the same task.

- The micro cuvettes (V_t = 0.7 ml) were cleaned by distilled water before each use and exterior surface of the cuvettes were wiped off before each reading in the spectrophotometer to get rid of any marks. Also, the cuvettes inverted several times by placing a piece of parafilm over to obtain a homogeneous solution and remove bubbles in the solution.

- The cuvette containing the blank solution (reaction mix without the sample) was placed to the reference cuvette holder to calibrate and auto zero the spectrophotometer.

- Enzyme activity was measured by detecting 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.

Amounts of enzyme are expressed as units because different enzymes have different catalytic capacities, there may be inactive (denatureted) molecules in a given mass and sometimes crude systems (e.g., tissue homogenates) may be used as enzyme source (Moorthy, 2008). The Enzyme Commission of the International Union of Biochemistry recommends expressing it in international units (IU) and defines 1 IU as the amount of an enzyme that catalyzes the transformation of 1 micromole of substrate per minute under standard conditions of temperature, optimal pH, and optimal substrate concentration. And specific activity relates activity to total mass of protein to avoid bias through individual differences of weight. Therefore, Pgm activity was expressed as units (micromoles of NADP reduced, per minute) per miligram of soluble protein at 25ºC .

2.2.3.3. Determination of the Glycogen Content

The glycogen molecule functions as the secondary long-term energy storage in animal cells and can be quickly mobilized to meet a sudden need for glucose. And we saw a correlation between PGM activity and allozyme polymorphism in preliminary experiments. To understand whether there is a correlation between allozyme polymorphism, PGM activity and glycogen amount, the samples that were used in the PGM activity measurements and allozyme studies were assayed spectrophotometrically to determine the glycogen content in each sample.

To make sure that glycogen concentration is within the range of the assay, several dilutions of the homogenates were experimented and the optimum dilution is determined to be 1:8 for adults and pupae and 1:3 for larvae. In the dilutions distilled water were used. The method used in this assay includes the measurement of free glucose emerged from the hydrolysis of glycogen by

Amyloglucosidase (exo-1,4-a-glucosidase; EC 3.2.1.3) that splits both 1,4- and 1,6-linkages in glycogen (Verrelli and Eanes, 2001).

To determine the actual concentration of glycogen in samples, standard curves were used with the help of absorbance readings of glycogen standards. When several dilutions of glycogen standards, with a known concentration, versus absorbance at their particular wavelength were plotted, the standard curves are obtained. According to these standard curve graphs the absorbance readings of the samples are converted into their corresponding glycogen concentrations.

R^2 values for the standard curves, the linear regression equation, the mean and standard deviation values were obtained for each block of samples. Actual glycogen concentrations of the samples were calculated according to the following formula:

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

After writing the equation of the straight line ($y = mx + b$), the equation for the concentration of the unknown sample becomes:

$$\left[\text{sample glycogen}\right] = (OD_{sample}\text{-}b)(1/m)(\text{dilution factor}).$$

Procedure:

- The reaction mix included one powder cap of glucose oxidase and peroxidase dissolved in 100 ml of distilled water, 1.6 ml of o-dianisidine dihydrochloride (50mg/ 20ml), and 10 units of amyloglucosidase. This mixture 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. This mixture was incubated in a 37ºC water bath for 30 minutes before its transfer on ice.

- Dilutions of glycogen standard and the samples were all loaded in duplicates. A schematic illustration of the loading seven dilutions of glycogen standard, blank and samples to 96-well microplate are given in Table 4.

- Multichannel pipette was used for loading of solutions to the microplates to avoid volumetric errors.

- Spectrophotometric measurements were performed at OD_{450} by using BioRad 680 microplate reader in flat-bottom 96-well microplates.

- Results were expressed as milligrams of glycogen per milligram of soluble protein.

Table 4. Representation of the 96-well microplates before the spectrophotometric measurements

	1	**2**	**3→→→12**
A	**Blank solution**	**Blank solution**	samples in duplicates
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. Determination of Total Soluble Protein Content

Within the context of this study concentrations of total soluble protein in the samples were measured to compare and correlate enzyme activity of PGM and glycogen content of different *Pgm* genotypes. Resulting data of enzyme activity and glycogen measurements were standardized by use of the total soluble protein content of the samples used in these assays. Therefore it was crucial to make accurate determination of protein concentration of these samples since enzyme activity and glycogen content data calculations could be erroneous because of any error in the protein content data.

A Bio-Rad protein assay (cat. no: 500-0002) was used in this study, for determining the actual concentration of total solubilized protein in homogenates. This protein assay is a dye-binding assay that uses the Coomassie G-250 dye which primarily binds to basic, aromatic or positively charged amino acid residues (lysine, histidine and especially arginine) in the acidic environment of the reagent and color change occurs in response to different concentrations of protein (Bradford, 1976; Campton and Jones,1985). The binding ratio of Coomassie dye to each protein molecule is approximately proportional to the number of positive charge in the protein (Bradley and Markwell, 2007). After the formation of the dye-protein complex, color of the solution gradually changes from reddish/brown to blue. Spectrophotometric measurement of the blue color can be performed at any wavelenght between 575 and 615 nm but, at the two extremes (at 575 or 615 nm) there will be a loss of measured color about 10% when compared to the one obtained at 595 nm (Bradleyl and Markwell, 2007).

As this protein assay employs Bradford method and an advantage of this method is that the absorbance of the dye-protein complex is stable relative to the other methods, critical timing is not essential for this procedure in such way that he color develops within 2 - 5 minutes and is stable up to 1-2 hours. Also

this method is quite sensitive that total protein concentration can be detected in the range of 0.2 to 1.4 mg of protein per ml (Bio-Rad Protein Assay Instruction Manual, 2007). But there can be some disadvantages of this method, such as a protein rich in arginine residues may lead to a artificially high result if Bradford assay is used or high concentrations of detergents will not be tolerated when this assay is used. (Bradley and Markwell, 2007; Bio-Rad Protein Assay Instruction Manual, 2009). Because of this reason, the compatibility of the sample protein and assay which is intented to be used is an important factor to get the more appropriate data.

Measurements were done at 570 nm with a microplate reader after the addition of acidic dye to the protein solution. Comparison of absorbances to a standard curve provides a relative protein concentrations in the homogenates. (Bio-Rad Protein Assay Instruction Manual, 2009).

Lyophilized bovine serum albumin standard was reconstituted with 20 ml of deionized water and mixed until dissolved. This standard solution was used up witin 30 days.

To determine the actual concentration of protein in samples, standard curves were used with the help of absorbance readings of BSA standards. When several dilutions of BSA and glycogen standards, with a known concentration, versus absorbance at their particular wavelength were plotted, the standard curves are obtained. According to these standard curve graphs the absorbance readings of the samples are converted into their corresponding protein concentrations.

R^2 values for the standard curves, the linear regression equation was obtained for each microplate of samples. Actual protein concentrations of the samples were calculated according to the following formula:

[(Sample OD-Y intercept) / (slope of the line)] (Dilution Factor)

After writing the equation of the straight line (y = mx + b), the equation for the concentration of the unknown sample becomes:

[sample protein] = (OD_{sample}-b)(1/m)(dilution factor).

Procedure for microtiter plates :

The procedure used in this study was optimized from Verrelli and Eanes (2001) and Bio-Rad Protein Assay Instruction Manual (2009).

- Dye reagent was prepared by diluting 1 volume of dye reagent concentrate with 4 volumes of distilled, deionized (DDI) water. Then, this dye reagent solution filtered through Whatmann # 1 filter to remove particulates and kept at room temperature in order to be used up in two weeks. After two weeks the dye reagent becomes heterogeneous and it should be discarded.

- Bovine serum albumin standard (Bio-Rad kit no: 500-0007) was prepared by diluting 20 ml of deionized water.

- Several dilutions of the sample homogenates were experimented to ensure that the protein concentration is within the range of the assay used and the optimum dilution was determined to be 1:6 for adults, 1:10 for pupae and 1:3 for larvae. Sample homogenates were diluted with distilled water.

- Seven dilutions of the protein standart, which is representative of the protein solution to be tested, were prepared within the linear range of 0.2 to 1.4 mg/ml for BSA. Although it is advised to prepare three to five dilutions of standards, we used seven dilutions to increase sensitivity and accuracy

- 5 µl of each standard and sample solution were pipetted into separate microplate wells. Proteins standards and homogenates were assayed in dublicate. A schematic illustration of the loading of BSA standard and sample homogenates to flat-bottom 96-well microplate is given in Table 5.

- 250 µl of diluted dye reagent was added to each well. Then, a multi-channel pipet used to dispense the reagent.

- The plates were incubated at room temperature for at least 10 minutes. Because absorbances would increase over time, samples weren't incubated for no more than 1 hour.

- After incubation, absorbances were measured at 570 nm in a BioRad plate reader. Absorbances were corrected for blank at OD_{570}.
- OD_{570} versus concentrations of the standards were plotted and relative protein concentrations of the samples were determined.

Table 5. Pattern of loading of the sample homogenates and BSA standards to the microplates in replicates

	1	**2**	**3→→→12**
A	**blank**	**blank**	samples in duplicates
B	BSA standard 0.2 mg/ml	BSA standard 0.2 mg/ml	
C	BSA standard 0.4 mg/ml	BSA standard 0.6 mg/ml	
D	BSA standard 0.6 mg/ml	BSA standard 0.6 mg/ml	
E	BSA standard 0.8 mg/ml	BSA standard 0.8 mg/ml	
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 1.45 mg/ml	BSA standard 1.45 mg/ml	

2.2.4. Statistical Analysis

2.2.4.1. Statistical Analysis of Allozyme Variation

The Hardy-Weinberg Principle has four basic assumptions: i) random mating; ii) allelic frequencies are conserved from generation to generation; ii) no significant migrations occur; and iv) mutation, genetic drift, selection and gene flow are negligible. All of these assumptions were assumed to be true before calculating expected genotypic frequencies and expected heterozygosities.

Allele frequencies are the basic genetic parameters of a population and give us the relative abundance of an allele in a population. Allele frequencies were calculated as the proportion of the observed allele to the total number of alleles in the population. This is formulated as:

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

Genotype frequency is the proportion of a particular genotype in a population. As another basic parameter, genotype frequencies are commonly used in explaining the genetic structure of a population. In this study, number of alleles, number of genotypes, allele and genotype frequencies at studied enzyme loci were calculated both for each month of sample collection and the for the total samples for all populations.

After determination of heterozygote and homozygote individuals by allozyme analysis, observed heterozygosity (H_O) is calculated with the formula below:

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

Expected heterozygosity (H_E) is computed based on known allelic frequencies and is an estimation of level of heterozygosity under Hardy-Weinberg equilibrium. Heterozygosities with Levene's correction which is the same as Nei's (1978) unbiased heterozygosity have been calculated by using POPGENE on the web software (Yeh and Yang, 1999). Comparison of observed and expected heterozygosity levels is an indicator of important population dynamics such as inbreeding.

Goodness-of-fit (G^2) tests were used to detect how close are the observed genotypic frequencies to those which would be expected under Hardy-Weinberg predictions. The null hypothesis (H_0) is that observed and expected heterozygosities are consistent with each other and the population is in Hardy-Weinberg proportions. Small probabilities (less than one percent) indicate a poor fit. Both of these calculations have been performed by using POPGENE software (Yeh and Yang, 1999).

2.2.4.2. Statistical Analysis of PGM Activity, Glycogen and Protein Content

All statistical analysis of biochemical data were performed via SPSS version 15.0 for Windows.

The relationship between each pair of biochemical data at every developmental stage was investigated using parametric test Pearson product-moment correlation. Preliminary analyses were performed to ensure no violation of assumptions of normality, linearity and homoscedasticity.

To avoid and minimize increased risk of inflated Type 1 error, MANOVA is preferred instead of ANOVA to compare means of groups. A one-way between groups multivariate analysis of variance (MANOVA) was performed to investigate differences among each of the independent variables of *A. mellifera* (developmental stages, subspecies, seasons and genotypes) in terms of biochemical data. Three independet variables were used: glycogen content, protein content and PGM activity. Preliminary assumption testing was conducted to check for normality, linearity, univariate and multivariate outliers, homogeneity of variance-covariance matrices, and multicollinearity, with no serious violations noted.

CHAPTER 3

RESULTS

3.1. Allozyme Data

3.1.1. Polymorphism at *Pgm, Hk, Pgi,* and *G6pd* Loci in *A. m. carnica* Population

A total number of 720 adult (18 month ×40 individuals per month) worker bees were sampled from Kırklareli for the electrophoretic analysis of *Pgm*, *Hk*, *Pgi*, and *G6pd* loci. The genotypes of all allozymes were named according to their relative mobility with the fastest allozyme used as the standard.

In *A. m. carnica* population, four alleles were observed in the electrophoretic analysis, namely *Pgm*-65, *Pgm*-75, *Pgm*-100 and *Pgm*-110, along 18 months from July 2008 to December 2009. *Pgm*-75 was the most common allele whereas *Pgm*-65 and *Pgm*-110 were the rare alleles (Table 8). The frequencies of common Pgm alleles differed seasonally as shown in Table 6. *Pgm* 65/65 and *Pg*m 110/110 genotypes have not been observed among studied samples and the frequencies of heterozygous genotypes for rare alleles were quite small.

Pgm 75/75 and *Pgm* 75/100 were the most common genotypes. Observed and expected frequencies of *Hk* genotypes, likelihood ratio test (G^2) and chi-square test (χ^2) values for Hardy-Weinberg equilibrium and corresponding probabilities for *A. m. carnica* population was given in Table 7. The frequencies of common *Pgm* genotypes differed seasonally. Also, the genotype

frequencies of spring and summer samples were found to be in Hardy-Weinberg equilibrium from April to September, whereas the genotype frequencies of winter samples exhibited significant levels of deviations from Hardy-Weinberg equilibrium.

Table 6. Allele frequencies at *Pgm* and *Hk* loci for *A. m. carnica* population.

Allele Frequencies of *Pgm* and *Hk*						
	Pgm-75	*Pgm-100*	*Pgm-110*	*Pgm-65*	*Hk-100*	*Hk-87*
July '08	0.9125	0.0875	0.0000	0.0000	0.9250	0.0750
Aug '08	1.0000	0.0000	0.0000	0.0000	0.9125	0.0875
Sept '08	0.8375	0.150	0.0000	0.0125	0.9250	0.0750
Oct '08	0.6250	0.3750	0.0000	0.0000	0.9500	0.050
Nov '08	0.5875	0.4000	0.0125	0.0000	0.9375	0.0625
Dec '08	0.5750	0.4000	0.0000	0.0250	0.9250	0.0750
Jan '09	0.5125	0.4875	0.0000	0.0000	0.9250	0.0750
Feb '09	0.5250	0.4750	0.0000	0.0000	0.9500	0.050
Mar '09	0.5125	0.4875	0.0000	0.0000	0.9250	0.750
Apr '09	0.7875	0.2000	0.0000	0.0125	0.9375	0.0625
May '09	0.8250	0.1750	0.0000	0.0000	0.9375	0.0625
June '09	0.8875	0.1000	0.0000	0.0125	0.9625	0.0375
July '09	0.9250	0.0750	0.0000	0.0000	0.9125	0.0875
Aug '09	0.9875	0.0125	0.0000	0.0000	0.9375	0.0625
Sept '09	0.8250	0.1625	0.0125	0.0000	0.9125	0.0875
Oct '09	0.6375	0.3500	0.0000	0.0125	0.9250	0.0750
Nov '09	0.5875	0.3875	0.0000	0.0250	0.9375	0.0625
Dec '09	0.5500	0.4500	0.000	0.000	0.9500	0.0500

Table 7. Observed and expected frequencies of common *Pgm* genotypes, likelihood ratio test (G^2) and chi-square test (χ^2) values for Hardy-Weinberg equilibrium and corresponding probabilities for *A. m. carnica* population.

Observed and Expected Frequencies of Common Pgm Genotypes								
	Observed			Expected				
	Pgm 75/100	*Pgm-75/75*	*Pgm 75/100*	*Pgm 75/75*	G^2	P	χ^2	P
July '08	0.175	0.825	0.832	0.162	0.217	0.642	0.312	0.577
Aug '08	0.000	1.000	-	-	-	-	-	-
Sept '08	0.300	0.675	0.254	0.700	2.344	0.504	1.376	0.711
Oct '08	0.750	0.250	0.475	0.388	18.672	0.000	13.849	0.000
Nov '08	0.800	0.175	0.476	0.342	24.890	0.000	19.049	0.000
Dec '08	0.800	0.150	0.466	0.328	27.403	0.000	21.139	0.000
Jan '09	0.925	0.050	0.506	0.260	33.575	0.000	28.129	0.000
Feb '09	0.950	0.050	0.505	0.273	41.233	0.000	31.843	0.000
Mar '09	0.975	0.025	0.506	0.259	46.479	0.000	35.243	0.000
Apr '09	0.400	0.575	0.319	0.618	4.374	0.224	2.716	0.438
May '09	0.350	0.650	0.292	0.679	2.780	0.095	1.656	0.198
June '09	0.200	0.775	0.180	0.786	1.017	0.797	0.565	0.904
July '09	0.150	0.850	0.141	0.855	0.406	0.524	0.217	0.642
Aug '09	0.025	0.975	0.025	0.975	0.000	1.000	0.000	1.000
Sept '09	0.325	0.650	0.272	0.679	2.780	0.427	1.655	0.647
Oct '09	0.700	0.275	0.452	0.403	16.948	0.001	12.419	0.006
Nov '09	0.775	0.175	0.461	0.342	24.890	0.000	19.049	0.000
Dec '09	0.850	0.125	0.501	0.299	23.0247	0.000	19.867	0.000

Table 8. Frequencies of rare *Pgm* genotypes for *A. m. carnica* population.

Frequencies of Rare Genotype Frequencies of *Pgm*				
	Pgm 100/100	*Pgm 65/75*	*Pgm 65/100*	*Pgm 75/110*
July ‘08	0.000	0.000	0.000	0.000
Aug ‘08	0.000	0.000	0.000	0.000
Sept ‘08	0.000	0.025	0.000	0.000
Oct ‘08	0.000	0.000	0.000	0.000
Nov ‘08	0.000	0.000	0.000	0.025
Dec ‘08	0.000	0.050	0.000	0.000
Jan ‘09	0.025	0.000	0.000	0.000
Feb ‘09	0.000	0.000	0.000	0.000
Mar ‘09	0.000	0.000	0.000	0.000
Apr ‘09	0.000	0.025	0.000	0.000
May ‘09	0.000	0.000	0.000	0.000
June ‘09	0.000	0.025	0.000	0.000
July ‘09	0.000	0.000	0.000	0.000
Aug ‘09	0.000	0.000	0.000	0.000
Sept ‘09	0.000	0.000	0.000	0.025
Oct ‘09	0.000	0.025	0.000	0.000
Nov ‘09	0.000	0.05	0.000	0.000
Dec ‘09	0.0250	0.000	0.000	0.000

The heterozygosity values of *A. m. carnica* population for each month of sampling were given in Table 9. There is a significant deviation from expected heterozygosities and Nei’s heterozygosities among winter samples from October ’08 to March ‘09, indicating that observed heterozygosity values exhibit a differentiation from the ones estimated under Hardy-Weinberg conditions. So this deviation among winter samples may be a sign of selection.

Table 9. Observed and expected heterozygosity values for *Pgm* locus.

Observed and Expected Heterezoygosity Values of *Pgm*			
	Observed	Expected*	Nei's Heterozygosity**
July '08	0.1750	0.1617	0.1597
Aug '08	0.00	0.00	0.00
Sept '08	0.3250	0.2794	0.2759
Oct '08	0.7500	0.4747	0.4688
Nov '08	0.8250	0.5009	0.4947
Dec '08	0.8500	0.5152	0.5088
Jan '09	0.9250	0.5060	0.4997
Feb '09	0.9500	0.5051	0.4988
Mar '09	0.9750	0.5060	0.4997
Apr '09	0.4250	0.3440	0.3397
May '09	0.3500	0.2924	0.2888
June '09	0.2250	0.2047	0.2022
July '09	0.1500	0.1405	0.1387
Aug '09	0.0250	0.0250	0.0247
Sept '09	0.3500	0.2965	0.2928
Oct '09	0.7250	0.4769	0.4709
Nov '09	0.8250	0.5104	0.5041
Dec '09	0.8500	0.5013	0.4950

* Expected heterozygosity were computed using Levene (1949)

** Nei's (1973) expected heterozygosity

In electrophoretic analysis of developmental stages we observed the same pattern of seasonal fluctuation of heterozygous genotype (*Pgm 75/100*) seen in adult samples at larval and pupal stages. Observed frequencies of *Pgm* genotypes, likelihood ratio test (G^2) values for Hardy-Weinberg equilibrium and corresponding probabilities for *A. m. carnica* population was given in Table 10. At each developmental stage summer samples are in Hardy-Weinberg equilibrium whereas winter samples exhibit significant deviations from Hardy-Weinberg equilibrium as seen in Table.

Table 10. Genotype frequencies, likelihood ratio test (G^2) for Hardy-Weinberg equilibrium and corresponding probabilities of winter and summer populations of developmental stages in *A. m. carnica* population.

Season			**Genotype Friquencies**		**G^2**	**p**	**$H_{Observed}$**	**$H_{Expected}$**
			Pgm 75/100	*Pgm 75/75*				
winter	larvae	carnica	0.8	0.2	5.14	0.02	0.80	0.5
winter	pupae	carnica	0.8	0.2	5.14	0.02	0.80	0.5
winter	adult	carnica	0.8	0.2	5.14	0.02	0.80	0.5
summer	larvae	carnica	0.1	0.9	0.00	1.00	0.10	0.10
summer	pupae	carnica	0.2	0.8	0.11	0.73	0.20	0.19
summer	adult	carnica	0.2	0.8	0.11	0.73	0.20	0.19

Seasonal variation in genotype frequencies of *Pgm* locus at each developmental stage were illustrated in Figure 5. However allozyme variants of PGM at egg stage could not be detected on starch gel, the same pattern of seasonal fluctuation were observed at larval, pupal and adult stages.

Two alleles were observed in the electrophoretic analysis of *A. m. carnica* population, namely *Hk*-87 and *Hk*-100. *Hk*-100 was the most common allele in this locus as shown in Table 6. *Hk* 87/100 and *Hk* 100/100 genotypes were observed in the electrophoresis. Observed and expected frequencies of *Hk* genotypes, likelihood ratio test (G^2) and chi-square test (χ^2) values for Hardy-Weinberg equilibrium and corresponding probabilities for *A. m. carnica* population was given in Table 11. In contrast to *Pgm* locus, the frequencies of *Hk* genotypes did not differed seasonally. Also, the genotype frequencies of all samples were found to be in Hardy-Weinberg equilibrium.

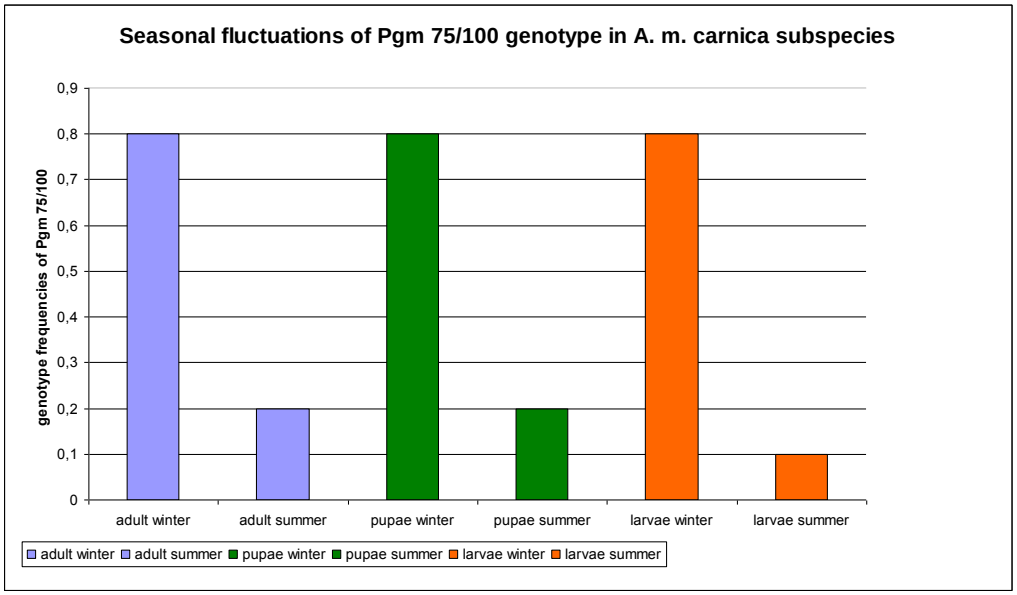


Figure 5. Seasonal fluctuations of *Pgm* 75/100 genotype in *A. m. carnica* subspecies

Not like the *Pgm* locus, the heterozygosity values remained stable throughout the year at *Hk* locus. The heterozygosity values for each month of sampling were given in Table 12. There is not a significant deviation from expected heterozygosities and Nei's heterozygosities among samples, indicating that observed heterozygosity values exhibit a differentiation from the ones estimated under Hardy-Weinberg conditions. So this deviation among winter samples may be a sign of selection.

Table 11. Observed and expected frequencies of common *Hk* genotypes, likelihood ratio test (G^2) and chi-square test (χ^2) values for Hardy-Weinberg equilibrium and corresponding probabilities for *A. m. carnica* population.

Genotype Frequencies of *Hk*								
	Observed		Expected					
	Hk *100/100*	*Hk* *87/100*	*Hk* *100/100*	*Hk* *87/100*	G^2	p	χ^2	P
July '08	0.85	0.15	0.8547	0.1405	0.4059	0.5241	0.2166	0.6416
Aug '08	0.825	0.175	0.8316	0.1617	0.5763	0.4477	0.3116	0.5767
Sept '08	0.85	0.15	0.8547	0.1405	0.4059	0.5241	0.2166	0.6416
Oct '08	0.90	0.10	0.9019	0.9620	0.1580	0.6910	0.0821	0.7745
Nov '08	0.875	0.125	0.8782	0.1187	0.2669	0.6054	0.1405	0.7077
Dec '08	0.85	0.15	0.8547	0.1405	0.4059	0.5241	0.2166	0.6416
Jan '09	0.85	0.15	0.8547	0.1405	0.4059	0.5241	0.2166	0.6416
Feb '09	0.90	0.10	0.9019	0.9620	0.1580	0.6910	0.0821	0.7745
Mar '09	0.85	0.15	0.8547	0.1405	0.4059	0.5241	0.2166	0.6416
Apr '09	0.875	0.125	0.8782	0.1187	0.2669	0.6054	0.1405	0.7077
May '09	0.875	0.125	0.8782	0.1187	0.2669	0.6054	0.1405	0.7077
June '09	0.925	0.075	0.926	0.728	0.0779	0.7800	0.0400	0.842
July '09	0.825	0.175	0.8316	0.1617	0.5763	0.4477	0.3116	0.5767
Aug '09	0.875	0.125	0.8782	0.1187	0.2669	0.6054	0.1405	0.7077
Sept '09	0.825	0.175	0.8316	0.1617	0.5763	0.4477	0.3116	0.5767
Oct '09	0.85	0.15	0.8547	0.1405	0.4059	0.5241	0.2166	0.6416
Nov '09	0.875	0.125	0.8782	0.1187	0.2669	0.6054	0.1405	0.7077
Dec '09	0.90	0.10	0.9019	0.9620	0.1580	0.6910	0.0821	0.7745

Table 12. Observed and expected heterozygosity values for *Hk* locus.

Observed and Expected Heterezoygosity Values of *Hk*			
	Observed	Expected	Nei's Heterozygosity
July '08	0.1500	0.1405	0.1387
Aug '08	0.1750	0.1617	0.1597
Sept '08	0.1500	0.1405	0.1387
Oct '08	0.1000	0.0962	0.0950
Nov '08	0.1250	0.1187	0.1172
Dec '08	0.1500	0.1405	0.1387
Jan '09	0.1500	0.1405	0.1387
Feb '09	0.1000	0.0962	0.0950
Mar '09	0.1500	0.1405	0.1387
Apr '09	0.1250	0.1187	0.1172
May '09	0.1250	0.1187	0.1172
June '09	0.0750	0.0731	0.0722
July '09	0.1750	0.1617	0.1597
Aug '09	0.1250	0.1187	0.1172
Sept '09	0.1750	0.1617	0.1597
Oct '09	0.1500	0.1405	0.1387
Nov '09	0.1250	0.1187	0.1172
Dec '09	0.1000	0.0962	0.0950

* Expected homozygosity and heterozygosity were computed using Levene (1949)

** Nei's (1973) expected heterozygosity

The temporal stability of *Hk* alleles frequencies and seasonal variation in allele frequencies of *Pgm* alleles were illustrated in Figure 6.

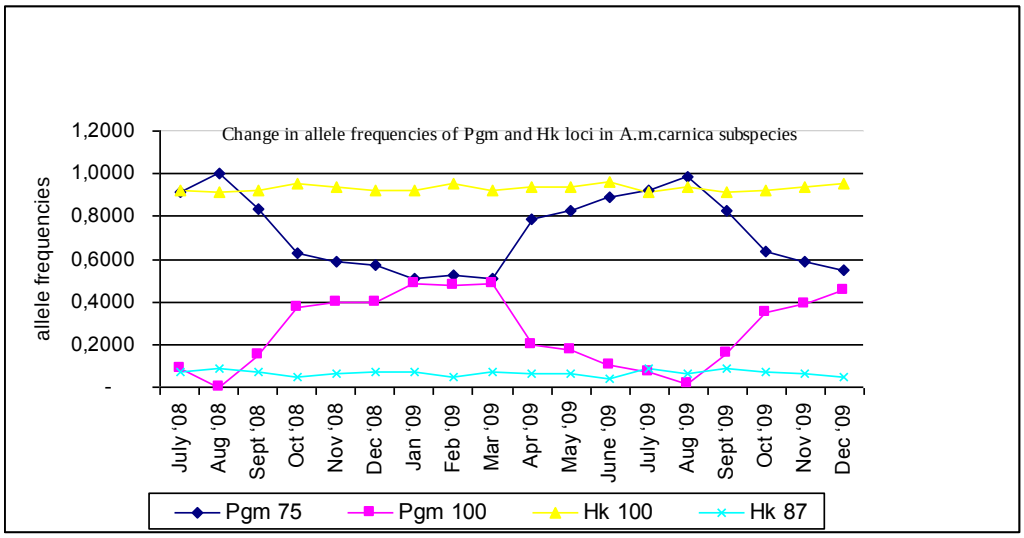


Figure 6. Change in allele frequencies of Pgm and Hk in *A. m. carnica* subspecies.

The temporal stability of *Hk* genotype frequencies and seasonal variation in genotype frequencies of *Pgm* alleles were illustrated in Figure 7.

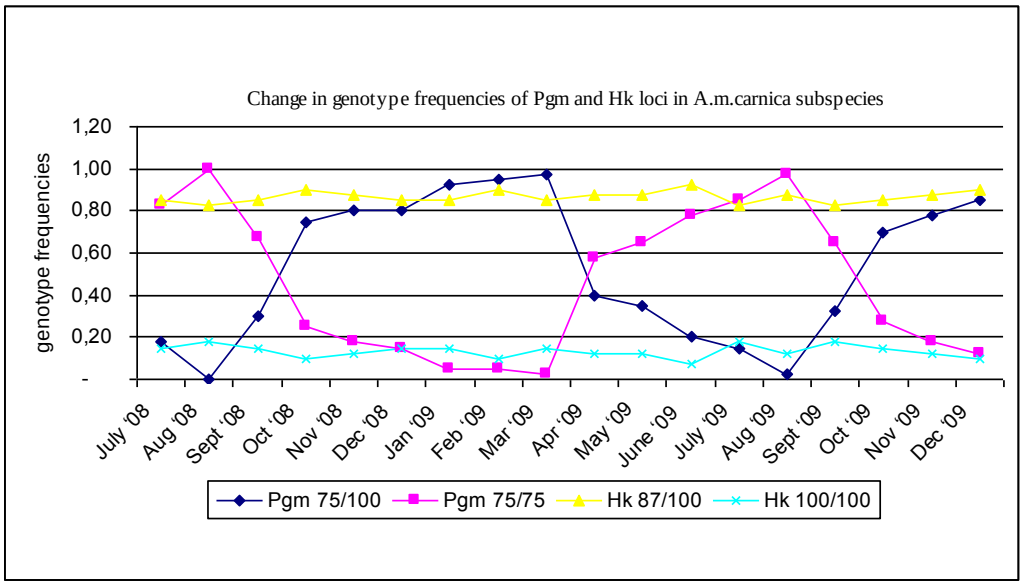


Figure 7. Change in genotype frequencies of *Pgm* and *Hk* loci in *A. m. carnica* subspecies.

In the *G6pd* and *Pgi* loci, no seasonal or temporal variation were detected and, *G6pd* 100 and *Pgi* 100 were the only alleles detected for these two loci.

3.1.2. Polymorphism at *Pgm, Hk, Pgi, and G6pd* Loci in *A. m. caucasica* Population

A total number of 720 adult (18 month ×40 individuals per month) worker bees were sampled from Artvin for the electrophoretic analysis of *Pgm*, *Hk*, *Pgi* and *G6pd* loci. The genotypes of all allozymes were named according to their relative mobility's with the fastest allozyme used as the standard.

Table 13. Allele frequencies at *Pgm* and *Hk* loci for *A. m. caucasica* population.

Allele Frequencies of Pgm and *Hk*						
	Pgm-75	*Pgm-100*	*Pgm-110*	*Pgm-65*	*Hk-100*	*Hk-87*
July '08	0.9250	0.0750	0.0000	0.0000	0.9375	0.0625
Aug '08	0.9875	0.0125	0.0000	0.0000	0.9125	0.0875
Sept '08	0.8120	0.1750	0.0000	0.0125	0.9250	0.0750
Oct '08	0.6625	0.3250	0.0000	0.0125	0.9375	0.0625
Nov '08	0.5375	0.4500	0.0125	0.0000	0.8875	0.1125
Dec '08	0.5375	0.4500	0.0125	0.0000	0.8875	0.1125
Jan '09	0.5375	0.4625	0.0000	0.0000	0.9250	0.0750
Feb '09	0.5000	0.5000	0.0000	0.0000	0.9375	0.0625
Mar '09	0.5125	0.4875	0.0000	0.0000	0.9500	0.0500
Apr '09	0.5500	0.4375	0.0000	0.0125	0.9250	0.0750
May '09	0.6875	0.3000	0.0125	0.000	0.9500	0.0500
June '09	0.8000	0.1875	0.0000	0.0125	0.9000	0.1000
July '09	0.9375	0.0625	0.0000	0.0000	0.9375	0.0625
Aug '09	1.0000	0.0000	0.0000	0.0000	0.9500	0.0500
Sept '09	0.6875	0.2875	0.0000	0.0250	0.9125	0.0875
Oct '09	0.6250	0.3625	0.0000	0.0125	0.9250	0.0750
Nov '09	0.5750	0.4250	0.0000	0.0000	0.9125	0.0875
Dec '09	0.5625	0.4250	0.0000	0.0125	0.9125	0,0875

In *A. m. caucasica* population, four alleles were observed in the electrophoretic analysis, namely *Pgm*-65, *Pgm*-75, *Pgm*-100 and *Pgm*-110, along 18 months from July 2008 to December 2009. *Pgm*-75 was the most common allele

whereas *Pgm*-65 and *Pgm*-110 were the rare alleles (Table 14). The frequencies of common *Pg*m alleles differed seasonally as shown in Table 13. *Pgm* 65/65 and *Pgm* 110/110 genotypes has not been observed among studied samples and the frequencies of heterozygous genotypes for rare alleles were quite small.

Table 14. Observed and expected frequencies of common *Pgm* genotypes, likelihood ratio test (G^2) and chi-square test (χ^2) values for Hardy-Weinberg equilibrium and corresponding probabilities for *A. m. caucasica* population.

Observed and Expected Frequencies of Common Genotype Frequencies of *Pgm*								
	Observed		Expected					
	Pgm 75/100	*Pgm-75/75*	*Pgm 75/100*	*Pgm 75/75*	G^2	P	χ^2	P
July '08	0.150	0.850	0.141	0.855	0.406	0.524	0.217	0.642
Aug '08	0.025	0.925	0.025	0.925	0.000	1.000	0.000	1.000
Sept '08	0.350	0.625	0.288	0.658	3.262	0.353	1.969	0.579
Oct '08	0.650	0.325	0.436	0.436	13.910	0.003	9.934	0.019
Nov '08	0.825	0.125	0.470	0.313	30.207	0.000	23.439	0.001
Dec '08	0.900	0.075	0.490	0.286	36.984	0.000	28.764	0.000
Jan '09	0.925	0.075	0.490	0.286	36.984	0.000	28.764	0.000
Feb '09	1.000	0.000	0.506	0.247	54.445	0.000	39.000	0.000
Mar '09	0.975	0.025	0.506	0.259	46.479	0.000	35.243	0.000
Apr '09	0.875	0.100	0.487	0.299	33.367	0.000	25.973	0.000
May '09	0.600	0.375	0.418	0.470	11.336	0.010	7.8790	0.049
June '09	0.375	0.600	0.304	0.638	3.792	0.285	2.3210	0.508
July '09	0.125	0.875	0.119	0.878	0.267	0.605	0.1410	0.708
Aug '09	0.000	1.000	-	-	-	-	-	-
Sept '09	0.575	0.375	0.400	0.470	11.336	0.010	7.8790	0.086
Oct '09	0.725	0.250	0.459	0.388	18.672	0.000	13.849	0.003
Nov '09	0.850	0.150	0.495	0.328	27.403	0.000	21.139	0.000
Dec '09	0.850	0.125	0.484	0.313	30.207	0.000	23.439	0.000

Pgm 75/75 and *Pg*m 75/100 were the most common genotypes. Observed and expected frequencies of *Pgm* genotypes, likelihood ratio test (G^2) and chi-square test (χ^2) values for Hardy-Weinberg equilibrium and corresponding probabilities for *A. m. caucasica* population was given in Table 14. The frequencies of common *Pgm* genotypes differed seasonally. Also, the genotype frequencies of spring and summer samples were found to be in Hardy-Weinberg equilibrium from May to September, whereas the genotype frequencies of winter samples exhibited significant levels of deviations from H-W equilibrium.

Table 15. Frequencies of rare *Pgm* genotypes in *A. m. caucasica* population.

Frequencies of Rare Genotype Frequencies of *Pgm*					
	Pgm-100/100	*Pgm*-65/65	*Pgm*-65/75	*Pgm*-65/100	*Pgm*-75/110
July ‘08	0.000	0.000	0.000	0.000	0.000
Aug ‘08	0.000	0.000	0.000	0.000	0.000
Sept ‘08	0.000	0.000	0.025	0.000	0.000
Oct ‘08	0.000	0.000	0.025	0.000	0.000
Nov ‘08	0.000	0.000	0.025	0.000	0.025
Dec ‘08	0.000	0.000	0.000	0.000	0.025
Jan ‘09	0.000	0.000	0.000	0.000	0.000
Feb ‘09	0.000	0.000	0.000	0.000	0.000
Mar ‘09	0.000	0.000	0.000	0.000	0.000
Apr ‘09	0.000	0.000	0.025	0.000	0.000
May ‘09	0.000	0.000	0.000	0.000	0.025
June ‘09	0.000	0.000	0.025	0.000	0.000
July ‘09	0.000	0.000	0.000	0.000	0.000
Aug ‘09	0.000	0.000	0.000	0.000	0.000
Sept ‘09	0.000	0.000	0.050	0.000	0.000
Oct ‘09	0.000	0.000	0.025	0.000	0.000
Nov ‘09	0.000	0.000	0.000	0.000	0.000
Dec ‘09	0.000	0.000	0.000	0.000	0.025

The heterozygosity values of *A. m. caucasica* population for each month of sampling were given in Table 16. There is a significant deviation from expected heterozygosities and Nei's heterozygosities among winter samples from October '08 to April '09, indicating that observed heterozygosity values exhibit a differentiation from the ones estimated under Hardy-Weinberg conditions. So this deviation among winter samples may be a sign of selection.

Table 16. Observed and expected heterozygosity values for *Pgm* locus in *A. m. caucasica* population

Observed and Expected Heterezoygosity Values of *Pgm*			
	Observed	Expected*	Nei's Heterozygosity**
July '08	0.1500	0.1405	0.1387
Aug '08	0.0250	0.0250	0.0247
Sept '08	0.3750	0.3130	0.3091
Oct '08	0.8750	0.5196	0.5131
Nov '08	0.9250	0.5149	0.5084
Dec '08	0.9250	0.5149	0.5084
Jan '09	0.9250	0.5035	0.4972
Feb '09	1.0000	0.5063	0.5000
Mar '09	0.9750	0.5060	0.4997
Apr '09	0.9000	0.5123	0.5059
May '09	0.6250	0.4427	0.4372
June '09	0.4000	0.3288	0.3247
July '09	0.1250	0.1187	0.1172
Aug '09	0.0000	0.0000	0.0000
Sept '09	0.6250	0.4497	0.4441
Oct '09	0.7500	0.4839	0.4778
Nov '09	0.8500	0.4949	0.4888
Dec '09	0.8750	0.5092	0.5028

* Expected heterozygosity were computed using Levene (1949)

** Nei's (1973) expected heterozygosity

In electrophoretic analysis of developmental stages we observed the same pattern of seasonal fluctuation of heterozygous and homozygous genotype

frequencies. seen in adult samples at larval and pupal stages. Observed frequencies of *Pgm* genotypes, likelihood ratio test (G^2) values for Hardy-Weinberg equilibrium and corresponding probabilities for *A. m. caucasica* population was given in Table 17. At each developmental stage summer samples are in Hardy-Weinberg equilibrium whereas winter samples exhibit significant deviations from Hardy-Weinberg equilibrium (Table 17).

Table 17. Genotype frequencies, likelihood ratio test (G^2) for Hardy-Weinberg equilibrium and corresponding probabilities of winter and summer populations of developmental stages in *A. m. caucasica* population.

Season			**Genotype Friquencies**		**G^2**	**p**	**$H_{Observed}$**	**$H_{Expected}$**
			Pgm 75/100	*Pgm 75/75*				
winter	larvae	caucasica	0.8	0.2	5.14	0.02	0.80	0.50
winter	pupae	caucasica	0.8	0.2	5.14	0.02	0.80	0.50
winter	adult	caucasica	0.7	0.3	3.43	0.06	0.70	0.48
summer	larvae	caucasica	0.2	0.8	0.11	0.73	0.20	0.19
summer	pupae	caucasica	0.2	0.8	0.11	0.73	0.20	0.19
summer	adult	caucasica	0.2	0.8	0.11	0.73	0.20	0.19

Seasonal variation in genotype frequencies of *Pgm* locus at each developmental stage were illustrated in Figure 8. However allozyme variants of *Pgm* at egg stage could not be detected on starch gel, the same pattern of seasonal fluctuation were observed at larval, pupal and adult stages.

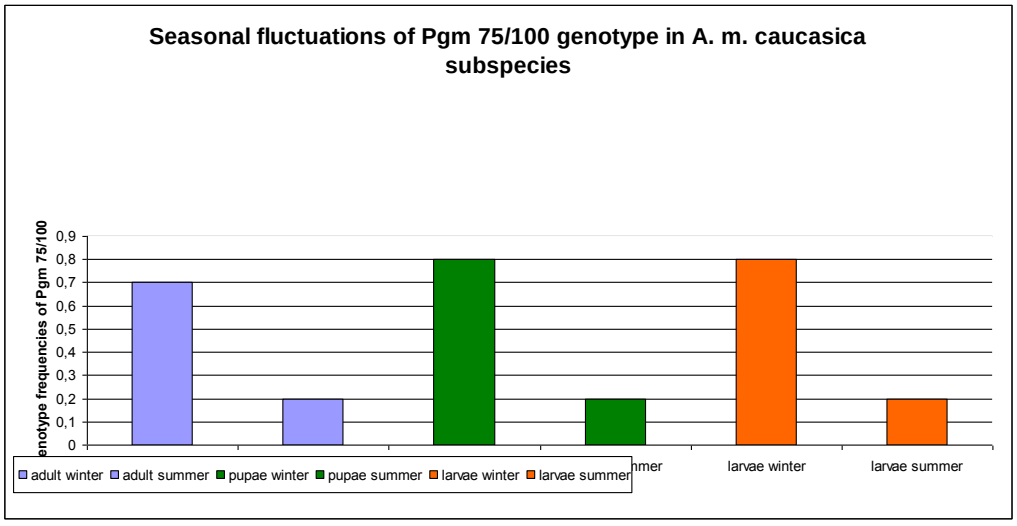


Figure 8. Seasonal fluctuations of *Pgm* 75/100 genotype in *A. m. caucasica* subspecies

Two alleles were observed in the electrophoretic analysis of *A. m. carnica* population, namely *Hk*-87 and *Hk*-100. *Hk*-100 was the most common allele in this locus as shown in Table 12. *Hk* 87/100 and *Hk* 100/100 genotypes were observed in the electrophoresis. Observed and expected frequencies of *Hk* genotypes, likelihood ratio test (G^2) and chi-square test (χ^2) values for Hardy-Weinberg equilibrium and corresponding probabilities for *A. m. carnica* population was given in Table 18. In contrast to *Pgm* locus, the frequencies of *Hk* genotypes did not differed seasonally. Also, the genotype frequencies of all samples were found to be in Hardy-Weinberg equilibrium.

Not like the *Pgm* locus, the heterozygosity values remained stable throughout the year at *Hk* locus. The heterozygosity values for each month of sampling were given in Table 19. There is not a significant deviation from expected heterozygosities and Nei's heterozygosities among samples, indicating that observed heterozygosity values exhibit a differentiation from the ones estimated under Hardy-Weinberg conditions. So this deviation among winter samples may be a sign of selection.

Table 18. Observed and expected frequencies of common *Hk* genotypes, likelihood ratio test (G^2) and chi-square test (χ^2) values for Hardy-Weinberg equilibrium and corresponding probabilities for *A. m. carnica* population.

Genotype Frequencies of *Hk*								
	Observed		Expected					
	Hk *100/100*	*Hk* *87/100*	*Hk* *100/100*	*Hk* *87/100*	G^2	P	χ^2	P
July '08	0.875	0.125	0.878	0.119	0.267	0.605	0.141	0.708
Aug '08	0.825	0.175	0.832	0.162	0.576	0.448	0.312	0.577
Sept '08	0.850	0.150	0.855	0.141	0.406	0.524	0.217	0.642
Oct '08	0.875	0.125	0.878	0.119	0.267	0.605	0.141	0.708
Nov '08	0.900	0.100	0.902	0.098	1.158	0.691	0.082	0.774
Dec '08	0.775	0.225	0.786	0.202	1.017	0.313	0.565	0.452
Jan '09	0.850	0.150	0.855	0.141	0.406	0.524	0.217	0.642
Feb '09	0.875	0.125	0.878	0.119	0.267	0.605	0.141	0.708
Mar '09	0.900	0.100	0.902	0.098	1.158	0.691	0.082	0.774
Apr '09	0.850	0.150	0.855	0.141	0.406	0.524	0.217	0.642
May '09	0.900	0.100	0.902	0.098	1.158	0.691	0.082	0.774
June '09	0.800	0.200	0.809	0.182	0.780	0.377	0.427	0.513
July '09	0.875	0.125	0.878	0.119	0.267	0.605	0.141	0.708
Aug '09	0.900	0.100	0.902	0.098	1.158	0.691	0.082	0.774
Sept '09	0.825	0.175	0.832	0.162	0.576	0.448	0.312	0.577
Oct '09	0.850	0.150	0.855	0.141	0.406	0.524	0.217	0.642
Nov '09	0.825	0.175	0.832	0.162	0.576	0.448	0.312	0.577
Dec '09	0.825	0.175	0.832	0.162	0.576	0.448	0.312	0.577

Table 19. Observed and expected heterozygosity values for *Hk* locus.

Observed and Expected Heterezoygosity Values of *Hk*			
	Observed	Expected	Nei's Heterozygosity
July '08	0.1250	0.1187	0.1172
Aug '08	0.1750	0.1617	0.1597
Sept '08	0.1500	0.1405	0.1387
Oct '08	0.1000	0.0962	0.0950
Nov '08	0.2250	0.2022	0.1997
Dec '08	0.2250	0.2022	0.1997
Jan '09	0.1500	0.1405	0.1387
Feb '09	0.1250	0.1187	0.1172
Mar '09	0.1000	0.0962	0.0950
Apr '09	0.1500	0.1405	0.1387
May '09	0.1000	0.0962	0.0950
June '09	0.2000	0.1823	0.1800
July '09	0.1250	0.1187	0.1172
Aug '09	0.1000	0.0962	0.0950
Sept '09	0.1750	0.1617	0.1597
Oct '09	0.1500	0.1405	0.1387
Nov '09	0.1750	0.1617	0.1597
Dec '09	0.1750	0.1617	0.1597

* Expected heterozygosity were computed using Levene (1949)

** Nei's (1973) expected heterozygosity

The temporal stability of *Hk* alleles frequencies and seasonal variation in allele frequencies of *Pgm* alleles in *A. m. caucasica* population were illustrated in Figure 9.

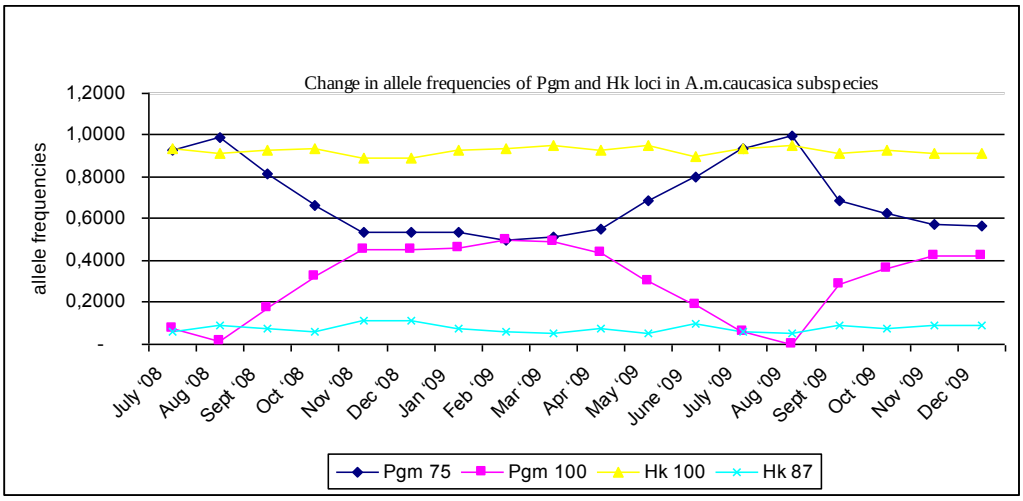


Figure 9. Change in allele frequencies of *Pgm* and *Hk* in *A. m. caucasica* subspecies.

The temporal stability of *Hk* genotype frequencies and seasonal variation in genotype frequencies of *Pgm* in *A. m. caucasica* population alleles were illustrated in Figure 10.

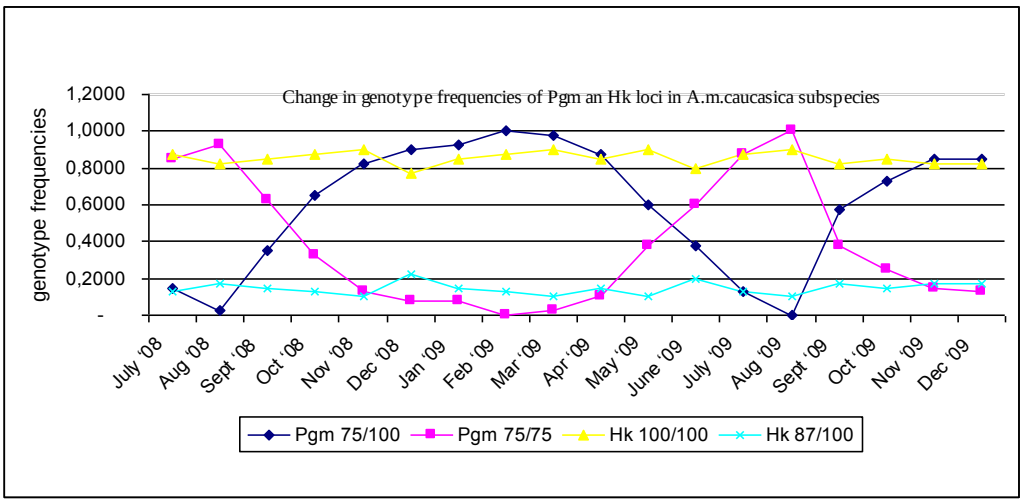


Figure 10. Change in genotype frequencies of *Pgm* and *Hk* in *A. m. caucasica* subspecies.

In the *G6pd* and *Pgi* loci, no seasonal or temporal variation were detected and, *G6pd* 100 and *Pgi* 100 were the only alleles detected for these two loci in *A. m. caucasica* population.

3.1.3. Polymorphism at *Pgm, Hk, Pgi, and G6pd* Loci in *A. m. syriaca* Population

A total number of 720 adult (18 month ×40 individuals per month) worker bees were sampled from Hatay for the electrophoretic analysis of *Pgm*, *Hk*, *Pgi* and *G6pd* loci. The genotypes of all allozymes were named according to their relative mobilities with the fastest allozyme used as the standard.

Table 20. Allele frequencies at *Pgm* and *Hk* loci for *A. m. syriaca* population.

Allele Frequencies of *Pgm* and *Hk*						
	Pgm-75	*Pgm-100*	*Pgm-110*	*Pgm-65*	*Hk-100*	*Hk-87*
July '08	0.9375	0.0625	0.0000	0.0000	0.9000	0.1000
Aug '08	0.9500	0.0500	0.0000	0.0000	0.9250	0.0750
Sept '08	0.8750	0.1250	0.0000	0.0000	0.8875	0.1125
Oct '08	0.8375	0.1625	0.0125	0.0000	0.9125	0.0875
Nov '08	0.6250	0.3750	0.0000	0.0000	0.9000	0.1000
Dec '08	0.5625	0.4375	0.000	0.000	0.9000	0.1000
Jan '09	0.5250	0.4750	0.000	0.000	0.8750	0.1250
Feb '09	0.5125	0.4825	0.000	0.000	0.9250	0.0750
Mar '09	0.5500	0.4500	0.000	0.000	0.9500	0.050
Apr '09	0.7750	0.2125	0.000	0.0125	0.9125	0.0875
May '09	0.8125	0.1625	0.0125	0.0125	0.9250	0.0750
June '09	.09000	0.1000	0.0000	0.0000	0.9500	0.0500
July '09	0.9500	0.0500	0.0000	0.0000	0.9250	0.0750
Aug '09	0.9625	0.0375	0.0000	0.0000	0.9125	0.0875
Sept '09	0.8875	0.1000	0.0000	0.0125	0.9000	0.1000
Oct '09	0.8625	0. 1375	0.0000	0.0000	0.9125	0.0875
Nov '09	0.6750	0.3250	0.0000	0.0000	0.8750	0.1250
Dec '09	0.6250	0.3625	0.0125	0.0000	0.9500	0.050

In *A. m. syriaca* population, four alleles were observed in the electrophoretic analysis, namely *Pgm*-65, Pgm-75, Pgm-100 and Pgm 110, along 18 months from July 2008 to December 2009. *Pgm*-75 was the most common allele

whereas *Pgm-65* and *Pgm-110* were the rare alleles (Table 22). The frequencies of common *Pgm* alleles differed seasonally as shown in Table 20. *Pgm* 65/65 and *Pgm* 110/110 genotypes has not been observed among studied samples and the frequencies of heterozygous genotypes for rare alleles were quite small.

Table 21. Observed and expected frequencies of common *Pgm* genotypes, likelihood ratio test (G^2) and chi-square test (χ^2) values for Hardy-Weinberg equilibrium and corresponding probabilities for *A. m. syriaca* population.

Observed and Expected Frequencies of Common Genotype Frequencies of *Pgm*								
	Observed			Expected				
	Pgm-75/100	*Pgm*-75/75	*Pgm*-75/100	*Pgm* 75/75	G^2	P	χ^2	P
July '08	0.125	0.875	0.119	0.878	0.267	0.605	0.141	0.708
Aug '08	0.100	0.900	0.096	0.902	0.158	0.691	0.082	0.774
Sept '08	0.250	0.750	0.764	0.222	1.290	0.256	0.727	0.394
Oct '08	0.300	0.700	0.300	0.700	0.000	0.988	0.000	0.988
Nov '08	0.750	0.250	0.475	0.388	18.672	0.000	13.849	0.000
Dec '08	0.875	0.125	0.498	0.313	30.207	0.000	23.439	0.000
Jan '09	0.950	0.050	0.505	0.272	41.233	0.000	31.843	0.000
Feb '09	0.975	0.025	0.506	0.259	46.479	0.000	35.243	0.000
Mar '09	0.900	0.100	0.501	0.299	33.367	0.000	25.973	0.000
Apr '09	0.425	0.550	0.334	0.598	5.011	0.171	3.155	0.368
May '09	0.325	0.625	0.267	0.658	3.262	0.775	1.969	0.923
June'09	0.200	0.800	0.182	0.809	0.800	0.377	0.427	0.513
July '09	0.100	0.900	0.096	0.902	0.158	0.691	0.082	0.774
Aug '09	0.075	0.925	0.073	0.926	0.078	0.780	0.040	0.842
Sept '09	0.200	0.775	0.180	0.786	1.017	0.797	0.565	0.904
Oct '09	0.275	0.725	0.240	0.742	1.602	0.206	0.914	0.339
Nov '09	0.650	0.350	0.444	0.453	12.571	0.000	8.857	0.003
Dec '09	0.725	0.250	0.459	0.388	18.672	0.000	13.849	0.003

Pgm 75/75 and *Pgm* 75/100 were the most common genotypes. Observed and expected frequencies of *Hk* genotypes, likelihood ratio test (G^2) and chi-square

test (χ^2) values for Hardy-Weinberg equilibrium and corresponding probabilities for *A. m. syriaca* population was given in Table 21. The frequencies of common *Pgm* genotypes differed seasonally. Also, the genotype frequencies of spring and summer samples were found to be in Hardy-Weinberg equilibrium from April '09 to October '09, whereas the genotype frequencies of winter samples exhibited significant levels of deviations from H-W equilibrium.

Table 22. Frequencies of rare *Pgm* genotypes for *A. m. syriaca* population.

Frequencies of Rare Genotype Frequencies of *Pgm*					
	Pgm 100/100	*Pgm-65/65*	*Pgm 65/75*	*Pgm 65/100*	*Pgm 75/110*
July '08	0.0000	0.0000	0.0000	0.0000	0.0000
Aug '08	0.0000	0.0000	0.0000	0.0000	0.0000
Sept '08	0.0000	0.0000	0.0000	0.0000	0.0000
Oct '08	0.025	0.0000	0.0000	0.0000	0.0000
Nov '08	0.0000	0.0000	0.0000	0.0000	0.0000
Dec '08	0.0000	0.0000	0.0000	0.0000	0.0000
Jan '09	0.0000	0.0000	0.0000	0.0000	0.0000
Feb '09	0.0000	0.0000	0.0000	0.0000	0.0000
Mar '09	0.0000	0.0000	0.0000	0.0000	0.0000
Apr '09	0.0000	0.0000	0.025	0.0000	0.0000
May '09	0.0000	0.0000	0.025	0.0000	0.025
June '09	0.0000	0.0000	0.0000	0.0000	0.0000
July '09	0.0000	0.0000	0.0000	0.0000	0.0000
Aug '09	0.0000	0.0000	0.0000	0.0000	0.0000
Sept '09	0.0000	0.0000	0.025	0.0000	0.0000
Oct '09	0.0000	0.0000	0.0000	0.0000	0.0000
Nov '09	0.0000	0.0000	0.0000	0.0000	0.0000
Dec '09	0.0000	0.0000	0.0000	0.0000	0.025

The heterozygosity values of *A. m. syriaca* population for each month of sampling were given in Table 23. There is a significant deviation from expected heterozygosities and Nei's heterozygosities among winter samples

from November ’08 to March ‘09, indicating that observed heterozygosity values exhibit a differentiation from the ones estimated under Hardy-Weinberg conditions. So this deviation among winter samples may be a sign of selection.

Table 23. Observed and expected heterozygosity values for *Pgm* locus.

Observed and Expected Heterezoygosity Values of *Pgm*			
	Observed	Expected*	Nei’s Heterozygosity**
July ‘08	0.1250	0.1187	0.1172
Aug ‘08	0.1000	0.0962	0.0950
Sept ‘08	0.2500	0.2215	0.2188
Oct ‘08	0.2750	0.2756	0.2722
Nov ‘08	0.7500	0.4747	0.4688
Dec ‘08	0.8750	0.4984	0.4922
Jan ‘09	0.9500	0.5051	0.4988
Feb ‘09	0.9750	0.5060	0.4997
Mar ‘09	0.9000	0.5013	0.4950
Apr ‘09	0.4500	0.3585	0.3541
May ‘09	0.3750	0.3171	0.3131
June ‘09	0.2000	0.1823	0.1800
July ‘09	0.1000	0.0962	0.0950
Aug ‘09	0.0750	0.0731	0.0722
Sept ‘09	0.2250	0.2047	0.2022
Oct ‘09	0.2750	0.2402	0.2372
Nov ‘09	0.6500	0.4443	0.4387
Dec ‘09	0.7500	0.4839	0.4778

* Expected heterozygosity were computed using Levene (1949)

** Nei's (1973) expected heterozygosity

In electrophoretic analysis of developmental stages we observed the same pattern of seasonal fluctuation of heterozygous genotype (*Pgm 75/100*) seen in adult samples at larval and pupal stages. Observed frequencies of *Pgm* genotypes, likelihood ratio test (G^2) values for Hardy-Weinberg equilibrium and corresponding probabilities for *A. m. syriaca* population was given in

Table 24. At each developmental stage summer samples are in Hardy-Weinberg equilibrium whereas winter samples exhibit significant deviations from Hardy-Weinberg equilibrium as seen in Table 24.

Table 24. Genotype frequencies, likelihood ratio test (G^2) for Hardy-Weinberg equilibrium and corresponding probabilities of winter and summer populations of developmental stages in *A. m. syriaca* population.

Season			**Genotype Friquencies**		**G^2**	**P**	**$H_{Observed}$**	**$H_{Expected}$**
			Pgm 75/100	*Pgm 75/75*				
winter	larvae	syriaca	0.9	0.1	7.71	0.01	0.9	0.52
winter	pupae	syriaca	0.9	0.1	7.71	0.01	0.9	0.52
winter	adult	syriaca	1.0	0,0	12.84	0.00	1.00	0.53
summer	larvae	syriaca	0.2	0.8	0.11	0.73	0.20	0.19
summer	pupae	syriaca	0.1	0.9	0.00	1.00	0.10	0.10
summer	adult	syriaca	0.1	0.9	0.00	1.00	0.10	0.10

Seasonal variation in genotype frequencies of *Pgm* locus at each developmental stage were illustrated in Figure 11. However allozyme variants of *Pgm* at egg stage could not be detected on starch gel, the same pattern of seasonal fluctuation were observed at larval, pupal and adult stages.

Two alleles were observed in the electrophoretic analysis of *A. m. carnica* population, namely *Hk*-87 and *Hk*-100. *Hk*-100 was the most common allele in this locus as shown in Table 18. *Hk* 87/100 and *Hk*100/100 genotypes were observed in the electrophoresis. Observed and expected frequencies of *Hk* genotypes, likelihood ratio test (G^2) and chi-square test (χ^2) values for Hardy-Weinberg equilibrium and corresponding probabilities for *A. m. carnica* population was given in Table 25. In contrast to *Pgm* locus, the frequencies of *Hk* genotypes did not differed seasonally. Also, the genotype frequencies of all samples were found to be in Hardy-Weinberg equilibrium.

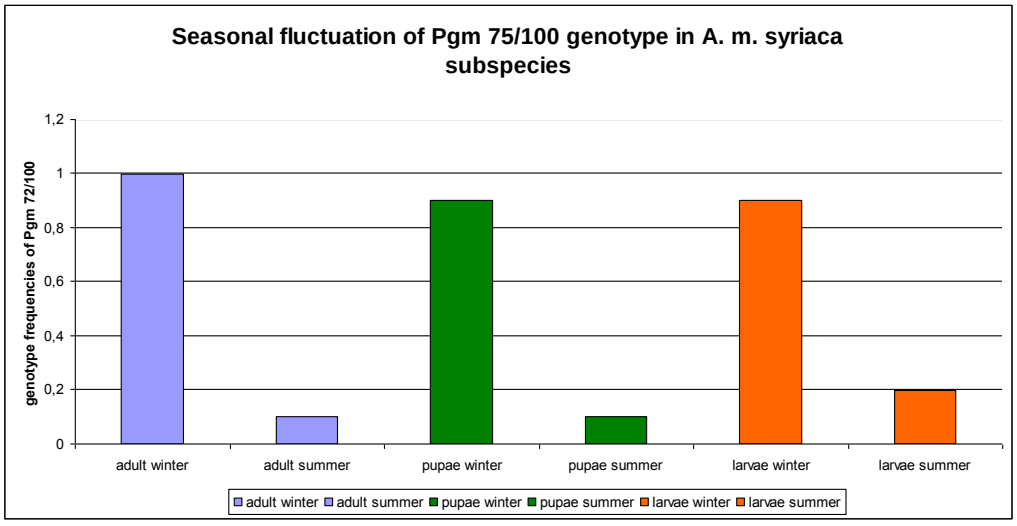


Figure 11. Seasonal fluctuations of *Pgm* 75/100 genotype frequency in *A. m. syriaca* subspecies

Not like the *Pgm* locus, the heterozygosity values remained stable throughout the year at *Hk* locus. The heterozygosity values for each month of sampling were given in Table 26. There is not a significant deviation from expected heterozygosities and Nei's heterozygosities among samples, indicating that observed heterozygosity values exhibit a differentiation from the ones estimated under Hardy-Weinberg conditions. So this deviation among winter samples may be a sign of selection.

Table 25. Observed and expected frequencies of common *Hk* genotypes, likelihood ratio test (G^2) and chi-square test (χ^2) values for Hardy-Weinberg equilibrium and corresponding probabilities for *A. m. carnica* population.

Genotype Frequencies of *Hk*								
	Observed		Expected					
	Hk-100/100	*Hk*-87/100	*Hk*-100/100	*Hk*-87/100	G^2	P	χ^2	P
July '08	0.800	0.200	0.809	0.182	0.880	0.377	0.427	0.513
Aug '08	0.850	0.150	0.855	0.141	0.406	0.524	0.217	0.642
Sept '08	0.775	0.225	0.786	0.202	1.017	0.313	0.565	0.452
Oct '08	0.825	0.175	0.832	0.162	0.576	0.448	0.312	0.577
Nov '08	0.800	0.200	0.809	0.182	0.880	0.377	0.427	0.513
Dec '08	0.800	0.200	0.809	0.182	0.880	0.377	0.427	0.513
Jan '09	0.750	0.250	0.764	0.222	1.290	0.256	0.727	0.394
Feb '09	0.850	0.150	0.855	0.141	0.406	0.524	0.217	0.642
Mar '09	0.900	0.100	0.902	0.096	0.158	0.691	0.082	0.774
Apr '09	0.825	0.175	0.832	0.162	0.576	0.448	0.312	0.577
May '09	0.850	0.150	0.855	0.141	0.406	0.524	0.217	0.642
June '09	0.900	0.100	0.902	0.096	0.158	0.691	0.082	0.774
July '09	0.850	0.150	0.855	0.141	0.406	0.524	0.217	0.642
Aug '09	0.825	0.175	0.832	0.162	0.576	0.448	0.312	0.577
Sept '09	0.800	0.200	0.809	0.182	0.880	0.377	0.427	0.513
Oct '09	0.825	0.175	0.832	0.162	0.576	0.448	0.312	0.577
Nov '09	0.750	0.250	0.764	0.222	1.290	0.256	0.727	0.394
Dec '09	0.900	0.100	0.902	0.096	0.158	0.691	0.082	0.774

Table 26. Observed and expected heterozygosity values for *Hk* locus.

Observed and Expected Heterezoygosity Values of *Hk*			
	Observed	Expected*	Nei's Heterozygosity**
July '08	0.2000	0.1823	0.1800
Aug '08	0.1500	0.1405	0.1387
Sept '08	0.2500	0.2022	0.1997
Oct '08	0.1750	0.1617	0.1597
Nov '08	0.2000	0.1823	0.1800
Dec '08	0.2000	0.1823	0.1800
Jan '09	0.2500	0.2215	0.2188
Feb '09	0.1500	0.1405	0.1387
Mar '09	0.1000	0.0962	0.0950
Apr '09	0.1750	0.1617	0.1597
May '09	0.1500	0.1405	0.1387
June '09	0.1000	0.0962	0.0950
July '09	0..1500	0.1405	0.1387
Aug '09	0.1750	0.1617	0.1597
Sept '09	0.2000	0.1823	0.1800
Oct '09	0.1750	0.1617	0.1 597
Nov '09	0.2500	0.2215	0.2188
Dec '09	0.1000	0.0962	0.0950

* Expected heterozygosity were computed using Levene (1949)

** Nei's (1973) expected heterozygosity

The temporal stability of *Hk* alleles frequencies and seasonal variation in allele frequencies of *Pgm* alleles were illustrated in Figure 12.

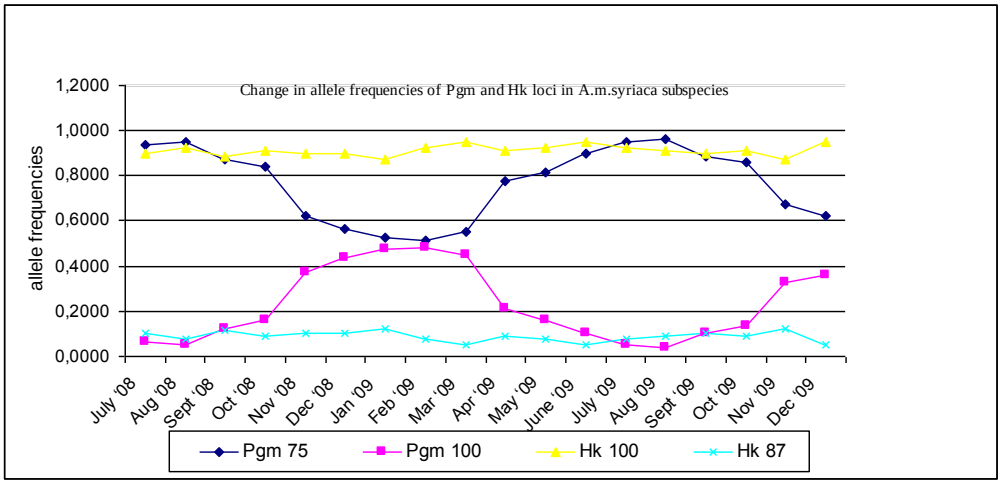


Figure 12. Change in allele frequencies of *Pgm* and *Hk* in *A. m. carnica* subspecies.

The temporal stability of *Hk* genotype frequencies and seasonal variation in genotype frequencies of *Pgm* alleles were illustrated in Figure 13.

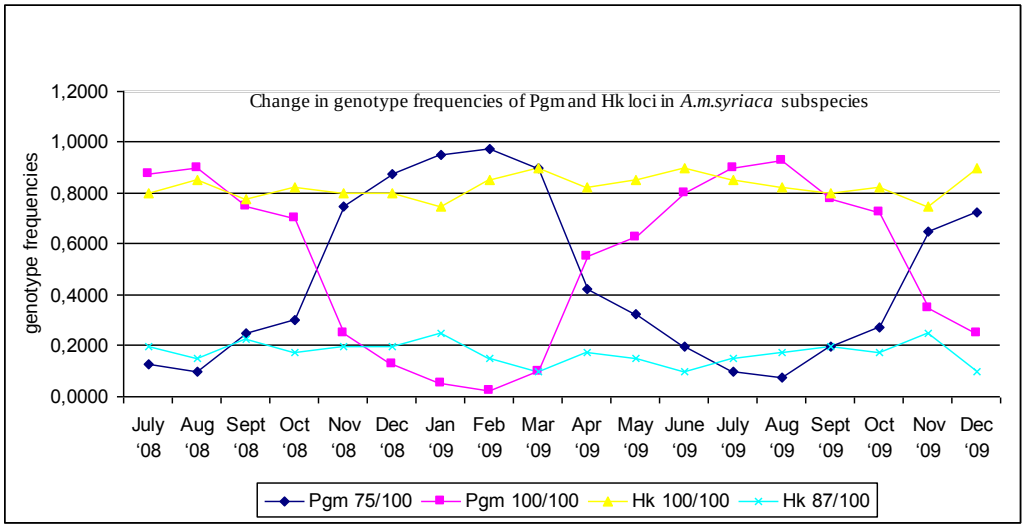


Figure 13. Change in genotype frequencies of *Pgm* and *Hk* in *A. m. caucasica* subspecies.

In the *G6pd* and *Pgi* loci, no seasonal or temporal variation were detected and, *G6pd* 100 and *Pgi* 100 were the only alleles detected for these two loci in *A. m. syriaca* population.

3.2. PGM Activity, Glycogen and Protein Content

A one-way between groups multivariate analysis of variance (MANOVA) was performed to investigate differences among developmental stages (adult, larva, pupa) of *A. mellifera* in biochemical data. Three independet variables were used: glycogen content, protein content and PGM activity. The independent variable was developmental stages of *A. mellifera* L. Preliminary assumption testing was conducted to check for normality, linearity, univariate and multivariate outliers, homogeneity of variance-covariance matrices, and multicollinearity, with no serious violations noted.

Table 27. Multivariate Tests(d) for developmental stages of *A. mellifera*.

Effect		Value	F	df	Sig.	Partial Eta Squared	Observed Power(a)
Intercept	Pillai's Trace	,944	986,041(b)	3	,000	,944	1,000
	Wilks' Lambda	,056	986,041(b)	3	,000	,944	1,000
	Hotelling's Trace	16,904	986,041(b)	3	,000	,944	1,000
	Roy's Largest Root	16,904	986,041(b)	3	,000	,944	1,000
stage	Pillai's Trace	1,404	138,128	6	,000	,702	1,000
	Wilks' Lambda	,087	139,357(b)	6	,000	,705	1,000
	Hotelling's Trace	4,848	140,584	6	,000	,708	1,000
	Roy's Largest Root	2,911	170,798(c)	3	,000	,744	1,000

a Computed using alpha = ,05

b Exact statistic

c The statistic is an upper bound on F that yields a lower bound on the significance level.

d Design: Intercept+stage

There is a statistically significant difference between developmental stages of *A. mellifera* on the combined dependent variables as shown in Table 27 (F=139.36 and p=0.000 for Wilks' Lambda=0.087, partial eta squared=0.705)

Table 28. Levene's Test of Equality of Error Variances(a) for developmental stages of *A. mellifera*.

	F	df1	df2	Sig.
glycogen	1,409	2	177	,247
activity	60,402	2	177	,000
protein	14,968	2	177	,000

Tests the null hypothesis that the error variance of the dependent variable is equal across groups.

a Design: Intercept+stage

Table 28 indicates that variances of enzyme activity and protein content data are not equal among developmental stages (F= 60,402 and p=0.00; F=14,968 and p=0.00 respectively) whereas variances of glycogen content data are equal among developmental stages (F=1.409, p=0.247).

Table 29. Tests of Between-Subjects Effects for developmental stages of *A. mellifera*.

Source	Dependent Variable	Type III Sum of Squares	df	Mean Square	F	Sig.	Partial Eta Squared	Observed Power(a)
Corrected Model	Glycogen	16,286(b)	2	8,143	17,354	,000	,164	1,000
	Activity	195696,119(c)	2	97848,060	194,590	,000	,687	1,000
	Protein	489,376(d)	2	244,688	199,899	,000	,693	1,000
Intercept	Glycogen	491,404	1	491,404	1047,236	,000	,855	1,000
	Activity	365071,724	1	365071,72	726,015	,000	,804	1,000
	Protein	1868,309	1	1868,309	1526,322	,000	,896	1,000
stage	Glycogen	16,286	2	8,143	17,354	,000	,164	1,000
	Activity	195696,119	2	97848,060	194,590	,000	,687	1,000
	Protein	489,376	2	244,688	199,899	,000	,693	1,000
Error	Glycogen	83,055	177	,469				
	Activity	89003,241	177	502,843				
	Protein	216,659	177	1,224				
Total	Glycogen	590,746	180					
	Activity	649771,084	180					
	Protein	2574,344	180					
Corrected Total	Glycogen	99,342	179					
	Activity	284699,360	179					
	Protein	706,035	179					

a Computed using alpha = ,05

b R Squared = ,164 (Adjusted R Squared = ,154)

c R Squared = ,687 (Adjusted R Squared = ,684)

d R Squared = ,693 (Adjusted R Squared = ,690)

When the results for the dependent variables were considered separately, each of the dependent data exhibit statistically significant difference among developmental stages (F= 194.59, p=0.00, partial eta squared= 0.687 for PGM activity; F=17.354, p=0.00, partial eta squared=0.164 for glycogen content;F=199.899, p=0.00, partial eta squared=0.693 for protein content) .

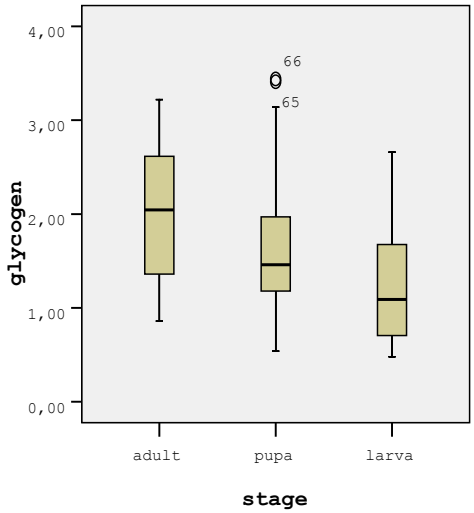


Figure 14. Glycogen content versus developmental stages of *A. mellifera* chart at adult stage (mean=2.01, st. dev= 0.72 for adults; mean=1.67, st.dev=0.7 for pupa; mean=1.28 st.dev=0.64 for larva)

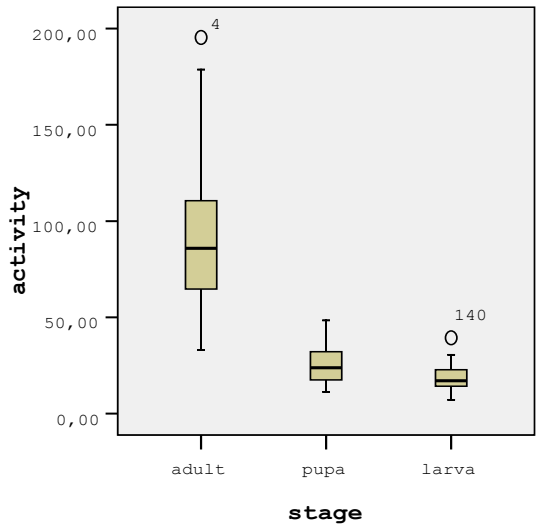


Figure 15. PGM activity versus developmental stages of *A. mellifera* chart at adult stage (mean=91.48, st. dev= 37.34 for adults; mean=25.42, st.dev=8.65 for pupa; mean=18.2 st.dev=6.29 for larva)

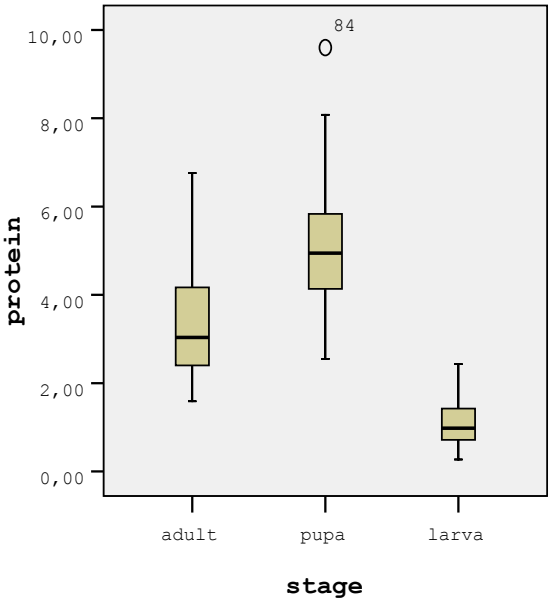


Figure 16. Protein content versus developmental stages of *A. mellifera* chart at adult stage (mean=3.44, st. dev= 1.28 for adults; mean=5.12, st.dev=1.31 for pupae; mean=1.10 st.dev=0.57 for larvae)

3.2.1. Adult Stage

3.2.1.1. Analysis of Protein Content

Protein measurements were performed to standardize PGM activity and glycogen content measurements so that these two types of data become comparable independent of individual weight differences. Absorbance readings measured at 570 nm and protein concentrations of the samples determined from their corresponding standard curves. In order to compare three subspecies in terms of protein content, 20 individuals from each of the subspecies were assayed as shown in Tables 30, 31, and 32.

Table 30. Protein contents of *A. m. carnica* at adult stage

	Winter			**Summer**	
		[Protein]			**[Protein]**
Sample	**Genotype**	**mg/mL**	**Sample**	**Genotype**	**mg/mL**
1	*Pgm 75/100*	6.76	11	*Pgm 75/75*	2.86
2	*Pgm 75/100*	2.14	12	*Pgm 75/75*	2.29
3	*Pgm 75/100*	3.89	13	*Pgm 75/75*	2.8
4	*Pgm 75/100*	2.94	14	*Pgm 75/75*	3.52
5	*Pgm 75/75*	5.64	15	*Pgm 75/100*	1.71
6	*Pgm 75/75*	5.98	16	*Pgm 75/75*	4.34
7	*Pgm 75/100*	2.29	17	*Pgm 75/100*	1.59
8	*Pgm 75/100*	2.63	18	*Pgm 75/75*	2.23
9	*Pgm 75/100*	3.66	19	*Pgm 75/75*	2.51
10	*Pgm 75/100*	3.14	20	*Pgm 75/75*	2.72
	Mean=3.91 SD=1.64			**Mean=2.66 SD=0.82**	

Table 31. Protein contents of *A. m. caucasica* at adult stage

Winter			Summer		
		[Protein]	Sample		[Protein]
Sample	**Genotype**	**mg/mg**		**Genotype**	**mg/mL**
1	*Pgm 75/75*	5.84	11	*Pgm 75/75*	2.54
2	*Pgm 75/100*	5.02	12	*Pgm 75/75*	3.63
3	*Pgm 75/75*	6.24	13	*Pgm 75/75*	2.67
4	*Pgm 75/100*	3.62	14	*Pgm 75/75*	4.20
5	*Pgm 75/100*	3.45	15	*Pgm 75/75*	4.14
6	*Pgm 75/75*	4.69	16	*Pgm 75/75*	2.26
7	*Pgm 75/100*	2.79	17	*Pgm 75/100*	1.88
8	*Pgm 75/100*	4.12	18	*Pgm 75/100*	1.96
9	*Pgm 75/100*	5.09	19	*Pgm 75/75*	2.28
10	*Pgm 75/100*	5.32	20	*Pgm 75/75*	1.93
	Mean=4.62 SD=1.10			**Mean=2.75 SD=0.91**	

Table 32. Protein contents of *A. m. syriaca* at adult stage

Winter			Summer		
Sample		**[Protein]**	**Sample**		**[Protein]**
	Genotype	**mg/mL**		**Genotype**	**mg/mL**
1	*Pgm 75/100*	3.8	11	*Pgm 75/100*	2.1
2	*Pgm 75/100*	4.24	12	*Pgm 75/75*	2.36
3	*Pgm 75/100*	5.69	13	*Pgm 75/75*	3.24
4	*Pgm 75/100*	2.19	14	*Pgm 75/75*	2.44
5	*Pgm 75/100*	3.08	15	*Pgm 75/75*	2.98
6	*Pgm 75/100*	3.79	16	*Pgm 75/75*	2.57
7	*Pgm 75/100*	2.77	17	*Pgm 75/75*	2.99
8	*Pgm 75/100*	5.42	18	*Pgm 75/75*	3.99
9	*Pgm 75/100*	4.34	19	*Pgm 75/75*	2.75
10	*Pgm 75/100*	3.96	20	*Pgm 75/75*	2.32
	Mean=3.83 SD=1.09			**Mean=2.77 SD=0.55**	

3.2.1.2. Analysis of Glycogen Content

Absorbance readings measured at 450 nm and glycogen concentrations of the samples determined from their corresponding standard curves. In order to compare three subspecies in terms of glycogen content, 20 individuals from each of the subspecies were assayed as shown in Tables 33, 34, and 35

Table 33 Glycogen contents of *A. m. carnica* at adult stage

Sample	**Winter Genotype**	**[Glycogen] mg/mg protein**	**Sample**	**Summer Genotype**	**[Glycogen] mg/mg protein**
1	*Pgm 75/100*	2.52	11	*Pgm 75/75*	1.91
2	*Pgm 75/100*	2.17	12	*Pgm 75/75*	1.05
3	*Pgm 75/100*	2.62	13	*Pgm 75/75*	1.52
4	*Pgm 75/100*	2.26	14	*Pgm 75/75*	1.22
5	*Pgm 75/75*	1.46	15	*Pgm 75/100*	2.66
6	*Pgm 75/75*	1.92	16	*Pgm 75/75*	0.86
7	*Pgm 75/100*	2.14	17	*Pgm 75/100*	2.61
8	*Pgm 75/100*	2.72	18	*Pgm 75/75*	1.50
9	*Pgm 75/100*	2.92	19	*Pgm 75/75*	1.22
10	*Pgm 75/100*	2.58	20	*Pgm 75/75*	1.12
	Mean=2.33 SD=0.43			**Mean=1.57 SD=0.63**	

Table 34. Glycogen contents of *A. m. caucasica* at adult stage

Sample	Winter Genotype	[Glycogen] mg/mg protein	Sample	Summer Genotype	[Glycogen] mg/mg protein
1	*Pgm 75/75*	1.90	11	*Pgm 75/75*	1.85
2	*Pgm 75/100*	2.54	12	*Pgm 75/75*	1.04
3	*Pgm 75/75*	1.33	13	*Pgm 75/75*	1.68
4	*Pgm 75/100*	2.60	14	*Pgm 75/75*	1.39
5	*Pgm 75/100*	2.43	15	*Pgm 75/75*	1.46
6	*Pgm 75/75*	1.95	16	*Pgm 75/75*	1.17
7	*Pgm 75/100*	2.39	17	*Pgm 75/100*	3.22
8	*Pgm 75/100*	2.99	18	*Pgm 75/100*	2.41
9	*Pgm 75/100*	2.32	19	*Pgm 75/75*	1.42
10	*Pgm 75/100*	2.26	20	*Pgm 75/75*	1.17
	Mean=2.27 SD=0.46			**Mean=1.68 SD=0.67**	

Table 35. Glycogen contents of *A. m. syriaca* at adult stage

Sample	Winter Genotype	[Glycogen] mg/mg protein	Sample	Summer Genotype	[Glycogen] mg/mg protein
1	*Pgm 75/100*	2.61	11	*Pgm 75/100*	2.85
2	*Pgm 75/100*	2.67	12	*Pgm 75/75*	1.46
3	*Pgm 75/100*	2.71	13	*Pgm 75/75*	1.42
4	*Pgm 75/100*	3.01	14	*Pgm 75/75*	1.11
5	*Pgm 75/100*	2.98	15	*Pgm 75/75*	1.14
6	*Pgm 75/100*	3.09	16	*Pgm 75/75*	1.05
7	*Pgm 75/100*	3.19	17	*Pgm 75/75*	1.14
8	*Pgm 75/100*	2.24	18	*Pgm 75/75*	1.61
9	*Pgm 75/100*	2.96	19	*Pgm 75/75*	1.03
10	*Pgm 75/100*	2.95	20	*Pgm 75/75*	1.05
	Mean=2.84 SD=0.28			**Mean=1.38 SD=0.55**	

3.2.1.3. Analysis of PGM Activity

Absorbance readings measured at 340 nm and enzyme activities of the samples determined according to the formula:

µmol of NADP reduced/min/ml = (ΔA-340 nm/min) X (0.6 ml assay volume/6.2) X (1/0.033 ml extract volume) X (1/d) X (dilution factor).

Specific activities were standardized by their corresponding protein amounts. In order to compare three subspecies in terms of enzyme activity, 20 individuals from each of the subspecies were assayed as shown in Tables 36, 37, and 38.

Table 36. PGM activities of *A. m. carnica* at adult stage.

Sample	**Winter Genotype**	**PGM activity mUnits/mg protein**	**Sample**	**Summer Genotype**	**PGM activity mUnits/mg protein**
1	*Pgm 75/100*	145.66	11	*Pgm 75/75*	67.15
2	*Pgm 75/100*	177.13	12	*Pgm 75/75*	96.99
3	*Pgm 75/100*	164.22	13	*Pgm 75/75*	55.46
4	*Pgm 75/100*	195.39	14	*Pgm 75/75*	54.55
5	*Pgm 75/75*	74.92	15	*Pgm 75/100*	110.35
6	*Pgm 75/75*	62.84	16	*Pgm 75/75*	33.1
7	*Pgm 75/100*	169.83	17	*Pgm 75/100*	116.58
8	*Pgm 75/100*	122.57	18	*Pgm 75/75*	91.36
9	*Pgm 75/100*	144.63	19	*Pgm 75/75*	77.18
10	*Pgm 75/100*	178.69	20	*Pgm 75/75*	71.82
	Mean=143.59 SD=44.52			**Mean=77.45 SD=26.46**	

Table 37. PGM activities of *A. m. caucasica* at adult stage.

Sample	Winter Genotype	PGM activity mUnits/mg protein	Sample	Summer Genotype	PGM activity mUnits/mg protein
1	*Pgm 75/75*	57.48	11	*Pgm 75/75*	69.03
2	*Pgm 75/100*	91.46	12	*Pgm 75/75*	65.33
3	*Pgm 75/75*	58.86	13	*Pgm 75/75*	70.05
4	*Pgm 75/100*	108.86	14	*Pgm 75/75*	46.52
5	*Pgm 75/100*	110.83	15	*Pgm 75/75*	36.70
6	*Pgm 75/75*	64.09	16	*Pgm 75/75*	67.25
7	*Pgm 75/100*	110.13	17	*Pgm 75/100*	95.97
8	*Pgm 75/100*	117.55	18	*Pgm 75/100*	87.75
9	*Pgm 75/100*	87.27	19	*Pgm 75/75*	70.84
10	*Pgm 75/100*	108.61	20	*Pgm 75/75*	40.66
	Mean=91.51 SD=23.54			**Mean=65.01 SD=19.13**	

Table 38. PGM activities of *A. m. syriaca* at adult stage.

Sample	Winter Genotype	PGM activity mUnits/mg protein	Sample	Summer Genotype	PGM activity mUnits/mg protein
1	*Pgm 75/100*	90.96	11	*Pgm 75/100*	89.86
2	*Pgm 75/100*	112.92	12	*Pgm 75/75*	73.59
3	*Pgm 75/100*	99.78	13	*Pgm 75/75*	50.51
4	*Pgm 75/100*	106.56	14	*Pgm 75/75*	65.69
5	*Pgm 75/100*	131.21	15	*Pgm 75/75*	74.53
6	*Pgm 75/100*	83.71	16	*Pgm 75/75*	55.47
7	*Pgm 75/100*	110.93	17	*Pgm 75/75*	73.21
8	*Pgm 75/100*	84.41	18	*Pgm 75/75*	47.29
9	*Pgm 75/100*	110.05	19	*Pgm 75/75*	55.26
10	*Pgm 75/100*	134.53	20	*Pgm 75/75*	62.62
	Mean=106.51 SD=17.52			**Mean=64.80 SD=13.19**	

3.2.1.4. Pearson Correlation of Biochemical Data at Adult Stage

Table 39. Pearson product-moment correlation of biochemical data of *A. m. carnica* subspecies at adult stage

		glycogen	activity	protein
glycogen	Pearson Correlation	1	,723(**)	-,031
	Sig. (2-tailed)		,000	,895
	N	20	20	20
activity	Pearson Correlation	,723(**)	1	-,155
	Sig. (2-tailed)	,000		,514
	N	20	20	20
protein	Pearson Correlation	-,031	-,155	1
	Sig. (2-tailed)	,895	,514	
	N	20	20	20

** Correlation is significant at the 0.01 level (2-tailed).

As far as *A. m. carnica* subspecies of adult stage is concerned, there is a positive strong correlation between glycogen content and PGM activity at the 0.01 significance level (r=0.723, p=0.00) as shown in Table 39. However, there is a negative weak correlation between protein content and PGM activity but this correlation is not statistically significant (r= -0.155, p= 0.514). Also, there is a negative weak correlation between protein content and glycogen content but this correlation is not statistically significant (r= -0.031, p= 0.895).

Table 40. Pearson product-moment correlation of biochemical data of *A. m. caucasica* subspecies at adult stage

		glycogen	activity	protein
glycogen	Pearson Correlation	1	,817(**)	,006
	Sig. (2-tailed)		,000	,981
	N	20	20	20
activity	Pearson Correlation	,817(**)	1	-,033
	Sig. (2-tailed)	,000		,889
	N	20	20	20
protein	Pearson Correlation	,006	-,033	1
	Sig. (2-tailed)	,981	,889	
	N	20	20	20

** Correlation is significant at the 0.01 level (2-tailed).

As far as *A. m. caucasica* subspecies of adult stage is concerned, there is a positive strong correlation between glycogen content and PGM activity at the 0.01 significance level (r=0.817, p=0.00) as shown in Table 40. However, there is a negative weak correlation between protein content and PGM activity but this correlation is not statistically significant (r= -0.033, p= 0.889). Also, there is a positive weak correlation between protein content and glycogen content but this correlation is not statistically significant (r= 0.005, p= 0.981).

As far as *A. m. syriaca* subspecies of adult stage is concerned, there is a positive strong correlation between glycogen content and PGM activity at the 0.01 significance level (r=0.838, p=0.00) as shown in Table 41. However, there is a positive weak correlation between protein content and PGM activity but this correlation is not statistically significant (r= 0.268, p= 0.253). Also, there is a positive medium correlation between protein content and glycogen content but this correlation is not statistically significant (r= 0.353, p= 0.127).

Table 41. Pearson product-moment correlation of biochemical data of *A. m. syriaca* subspecies at adult stage

		glycogen	activity	protein
glycogen	Pearson Correlation	1	,838(**)	,353
	Sig. (2-tailed)		,000	,127
	N	20	20	20
activity	Pearson Correlation	,838(**)	1	,268
	Sig. (2-tailed)	,000		,253
	N	20	20	20
protein	Pearson Correlation	,353	,268	1
	Sig. (2-tailed)	,127	,253	
	N	20	20	20

** Correlation is significant at the 0.01 level (2-tailed).

3.2.1.5. MANOVA Results for Biochemical Data at Adult Stage

3.2.1.5.1. Subspecies vs. Biochemical Data

A one-way between groups multivariate analysis of variance (MANOVA) was performed to investigate subspecies differences in biochemical data. Three independet variables were used: glycogen content, protein content and PGM activity. The independent variable was subspecies of *A. mellifera* L. Preliminary assumption testing was conducted to check for normality, linearity, univariate and multivariate outliers, homogeneity of variance-covariance matrices, and multicollinearity, with no serious violations noted.

There is a statistically significant difference between subspecies of *A. mellifera* on the combined dependent variables of adult stage as shown in Table 39 (F=3.6 and p=0.003 for Wilks' Lambda=0.7, partial eta squared=0.164)

Table 42. Multivariate Tests(d) for subspecies at adult stage.

Effect		Value	F	df	Sig.	Partial Eta Squared	Observed Power(a)
Intercept	Pillai's Trace	,941	290,538(b)	3	,000	,941	1,000
	Wilks' Lambda	,059	290,538(b)	3	,000	,941	1,000
	Hotelling's Trace	15,848	290,538(b)	3	,000	,941	1,000
	Roy's Largest Root	15,848	290,538(b)	3	,000	,941	1,000
subsp	Pillai's Trace	,307	3,383	6	,004	,153	,930
	Wilks' Lambda	,699	3,600(b)	6	,003	,164	,945
	Hotelling's Trace	,423	3,810	6	,002	,175	,957
	Roy's Largest Root	,404	7,536(c)	3	,000	,288	,981

a Computed using alpha = ,05

b Exact statistic

c The statistic is an upper bound on F that yields a lower bound on the significance level.

d Design: Intercept+subsp

Table 43. Levene's Test of Equality of Error Variances(a)
for subspecies at adult stage.

	F	df1	df2	Sig.
glycogen	4,898	2	57	,011
activity	9,368	2	57	,000
protein	,994	2	57	,377

Tests the null hypothesis that the error variance of the dependent variable is equal across groups.

a Design: Intercept+subsp

Table 43 indicates that variances of glycogen content and enzyme activity data are different among subspecies (F= 4,898 and p=0.011; F=9,368 and p=0.00 respectively) whereas variances of protein data similar among subspecies at adult stage (F=0.994, p=0.377).

When the results for the dependent variables were considered separately, the only difference to reach statistical significance is PGM activity (F= 4.597, p=0.014, partial eta squared= 0.139). This means that 13.9 per cent of the variance in enzyme activity data are explained by subspecies of *A. mellifera* at adult stage as shown in Table 44.

Table 44. Tests of Between-Subjects Effects for subspecies at adult stage.

Source	Dependent Variable	Type III Sum of Squares	df	Mean Square	F	Sig.	Partial Eta Squared	Observed Power(a)
Corrected Model	glycogen	,311(b)	2	,156	,296	,745	,010	,095
	Activity	11424,233(c)	2	5712,117	4,597	,014	,139	,757
	Protein	1,843(d)	2	,922	,558	,575	,019	,138
Intercept	glycogen	243,090	1	243,090	462,940	,000	,890	1,000
	Activity	502106,276	1	502106,276	404,109	,000	,876	1,000
	Protein	709,534	1	709,534	429,510	,000	,883	1,000
subsp	glycogen	,311	2	,156	,296	,745	,010	,095
	Activity	11424,233	2	5712,117	4,597	,014	,139	,757
	Protein	1,843	2	,922	,558	,575	,019	,138
Error	glycogen	29,931	57	,525				
	Activity	70822,546	57	1242,501				
	Protein	94,162	57	1,652				
Total	glycogen	273,332	60					
	Activity	584353,055	60					
	Protein	805,540	60					
Corrected Total	glycogen	30,242	59					
	Activity	82246,779	59					
	Protein	96,005	59					

a Computed using alpha = ,05

b R Squared = ,010 (Adjusted R Squared = -,024)

c R Squared = ,139 (Adjusted R Squared = ,109)

d R Squared = ,019 (Adjusted R Squared = -,015)

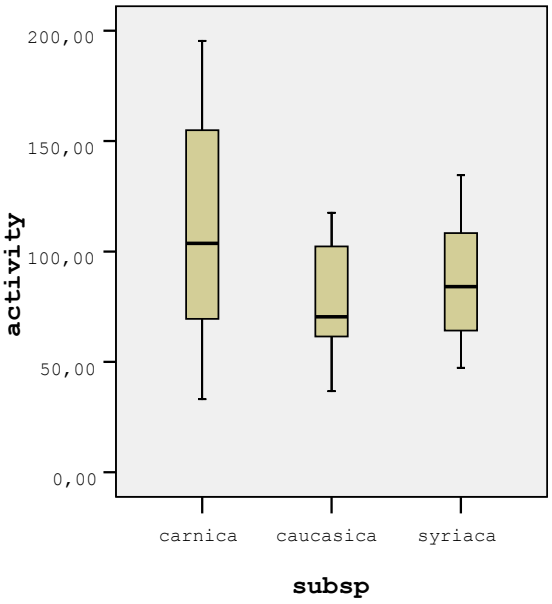


Figure 17. PGM activity versus subspecies of *A. mellifera* chart at adult stage (mean=110.52, st. dev= 49.21 for *A. m. carnica* ; mean=78.26, st.dev=24.91 for *A. m. caucasica* ; mean=85.65 st.dev=26.18 for *A. m. syriaca).*

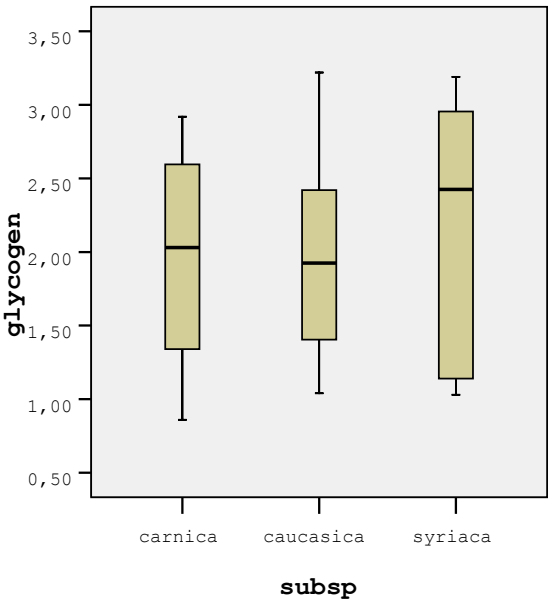


Figure 18. Glycogen content versus subspecies of *A. mellifera* chart at adult stage (mean=1.949, st. dev= 0.65725 for *A. m. carnica* ; mean=1.976, st.dev=0.635 for *A. m. caucasica* ; mean=2.114 st.dev=0.860 for *A. m. syriaca*).

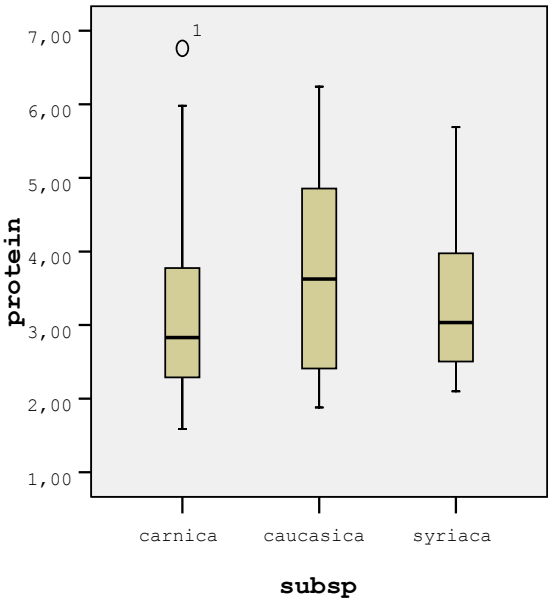


Figure 19. Protein content versus subspecies of *A. mellifera* chart at adult stage (mean=3.28, st. dev= 1.42 for *A. m. carnica* ; mean=3.68, st.dev=1.37 for *A. m. caucasica* ; mean=3.35 st.dev=1.03 for *A. m. syriaca*)

3.2.1.5.2. MANOVA for Season vs. Biochemical Data

A one-way between groups multivariate analysis of variance (MANOVA) was performed to investigate seasonal differences in biochemical data. Three independet variables were used: glycogen content, protein content and PGM activity. The independent variable was season. Preliminary assumption testing was conducted to check for normality, linearity, univariate and multivariate outliers, homogeneity of variance-covariance matrices, and multicollinearity, with no serious violations noted.

There is a statistically significant difference between seasons on the combined dependent variables at adult stage as shown in Table 45 (F=71.58, p=0.00, Wilks' Lambda=0.21, partial eta squared=0.793)

Table 45. Multivariate Tests(d) for season at adult stage.

Effect		Value	F	df	Sig.	Partial Eta Squared	Observed Power(a)
Intercept	Pillai's Trace	,987	1412,416(b)	3	,000	,987	1,000
	Wilks' Lambda	,013	1412,416(b)	3	,000	,987	1,000
	Hotelling's Trace	75,665	1412,416(b)	3	,000	,987	1,000
	Roy's Largest Root	75,665	1412,416(b)	3	,000	,987	1,000
season	Pillai's Trace	,793	71,577(b)	3	,000	,793	1,000
	Wilks' Lambda	,207	71,577(b)	3	,000	,793	1,000
	Hotelling's Trace	3,834	71,577(b)	3	,000	,793	1,000
	Roy's Largest Root	3,834	71,577(b)	3	,000	,793	1,000

a Computed using alpha = ,05

b Exact statistic

c Design: Intercept+season

Table 46. Levene's Test of Equality of Error Variances(a) for seasons at adult stage.

	F	df1	df2	Sig.
glycogen	1,149	1	58	,288
activity	7,276	1	58	,009
protein	11,140	1	58	,001

Tests the null hypothesis that the error variance of the dependent variable is equal across groups.

a Design: Intercept+season

Table 46 indicates that variances of protein content and enzyme activity data are not equal among seasons (F= 7,276 and p=0.009; F=11,140 and p=0.01 respectively) whereas variances of glycogen data are equal among subspecies at adult stage (F=1.149, p=0.288).

When the results for the dependent variables were considered seperately, glycogen content, PGM activity and protein content data exhibit statistically significant difference between seasons at adult stage (F= 44.628, p=0.00, partial eta square=0.435; F=33.442, p=0.00, partial eta square= 0.366;

F=26.916, p=0.00, partial eta square=0.317 respectively) This means that 43.5 per cent of the variance in glycogen content data, 36.6 per cent of the variance in enzyme activity data and 31.7 per cent of the variance in protein content data are explained by seasonal differentiation as shown in Table 47.

Table 47. Tests of Between-Subjects Effects for seasons at adult stage.

Source	Dependent Variable	Type III Sum of Squares	df	Mean Square	F	Sig.	Partial Eta Squared	Observed Power(a)
Corrected Model	Glycogen	13,151(b)	1	13,151	44,628	,000	,435	1,000
	Activity	30079,174(c)	1	30079,174	33,442	,000	,366	1,000
	Protein	30,431(d)	1	30,431	26,916	,000	,317	,999
Intercept	Glycogen	243,090	1	243,090	824,939	,000	,934	1,000
	Activity	502106,276	1	502106,276	558,242	,000	,906	1,000
	Protein	709,534	1	709,534	627,576	,000	,915	1,000
season	Glycogen	13,151	1	13,151	44,628	,000	,435	1,000
	Activity	30079,174	1	30079,174	33,442	,000	,366	1,000
	Protein	30,431	1	30,431	26,916	,000	,317	,999
Error	Glycogen	17,091	58	,295				
	Activity	52167,605	58	899,441				
	Protein	65,575	58	1,131				
Total	Glycogen	273,332	60					
	Activity	584353,055	60					
	Protein	805,540	60					
Corrected Total	Glycogen	30,242	59					
	Activity	82246,779	59					
	Protein	96,005	59					

a Computed using alpha = ,05

b R Squared = ,435 (Adjusted R Squared = ,425)

c R Squared = ,366 (Adjusted R Squared = ,355)

d R Squared = ,317 (Adjusted R Squared = ,305)

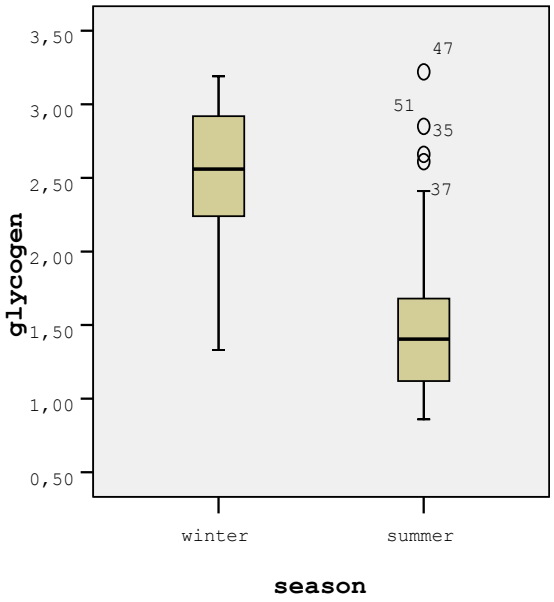


Figure 20. Glycogen content versus season chart at adult stage (mean=2.48, st. dev= 0.46 for winter; mean=1.54 st.dev=0.61 for summer)

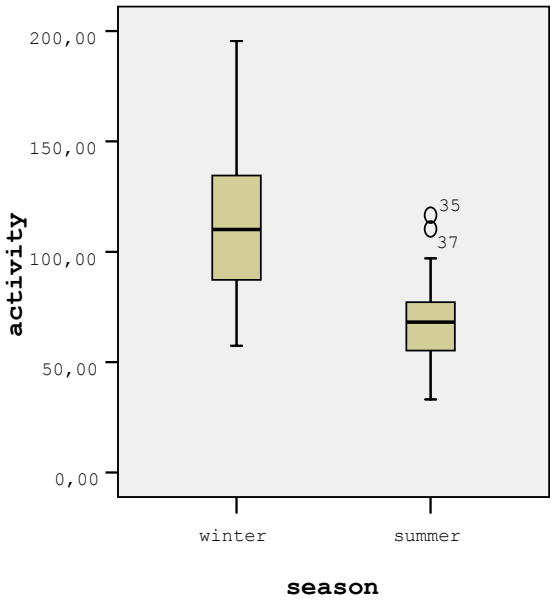


Figure 21. PGM activity versus season chart at adult stage (mean=111.9, st. dev= 37.12 for winter; mean=69.09 st.dev=20.52 for summer).

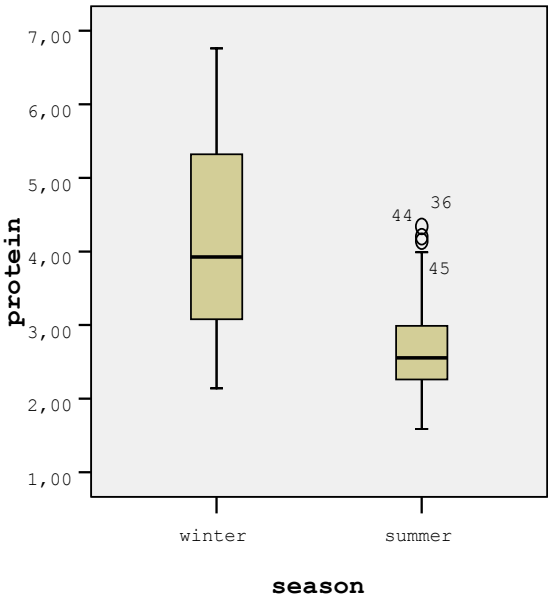


Figure 22. Protein content versus season chart at adult stage (mean=4.15, st. dev= 1.30 for winter; mean=2.72 st.dev=0.74 for summer).

3.2.1.5.3. MANOVA Results for Genotype vs. Biochemical Data

A one-way between groups multivariate analysis of variance (MANOVA) was performed to investigate genotypic differences in biochemical data at adult stage. Three independet variables were used: glycogen content, protein content and PGM activity. The independent variable was genotype. Preliminary assumption testing was conducted to check for normality, linearity, univariate and multivariate outliers, homogeneity of variance-covariance matrices, and multicollinearity, with no serious violations noted.

There is a statistically significant difference between genotypes of *Pgm* allozymes on the combined dependent variables at adult stage as shown in Table 48 (F=130, p=0.00, Wilks' Lambda=0.126, partial eta squared=0.874).

Table 48. Multivariate Tests (d) for genotypes at adult stage.

Effect		Value	F	df	Sig.	Partial Eta Squared	Observed Power(a)
Intercept	Pillai's Trace	,987	1450,022(b)	3	,000	,987	1,000
	Wilks' Lambda	,013	1450,022(b)	3	,000	,987	1,000
	Hotelling's Trace	77,680	1450,022(b)	3	,000	,987	1,000
	Roy's Largest Root	77,680	1450,022(b)	3	,000	,987	1,000
genotype	Pillai's Trace	,874	129,394(b)	3	,000	,874	1,000
	Wilks' Lambda	,126	129,394(b)	3	,000	,874	1,000
	Hotelling's Trace	6,932	129,394(b)	3	,000	,874	1,000
	Roy's Largest Root	6,932	129,394(b)	3	,000	,874	1,000

a Computed using alpha = ,05

b Exact statistic

c Design: Intercept+genotype

Table 49. Levene's Test of Equality of Error Variances(a) for genotypes at adult stage.

	F	df1	df2	Sig.
glycogen	,028	1	58	,867
activity	12,523	1	58	,001
protein	,088	1	58	,768

Tests the null hypothesis that the error variance of the dependent variable is equal across groups.

a Design: Intercept+genotype

Table 49 indicates that variances of enzyme activity data are not equal among subspecies (F= 12,523 and p=0.001) whereas variances of glycogen content and protein content data are equal between genotypes at adult stage (F=0.028, p=0.867; F=0.088, p=768 respectively).

When the results for the dependent variables were considered separately, glycogen content and PGM activity data exhibit statistically significant difference between genotypes at adult stage (F= 256.550, p=0.00, partial eta square=0.816; F=83.877, p=0.00, partial eta square= 0.591). This means that 81.6 per cent of the variance in glycogen content data and 59.1 per cent of the

variance in enzyme activity data and 31.7 per cent of the variance in protein content data are explained by seasonal differentiation as shown in Table 50.

Table 50. Tests of Between-Subjects Effects for genotypes at adult stage.

Source	Dependent Variable	Type III Sum of Squares	df	Mean Square	F	Sig.	Partial Eta Squared	Observed Power(a)
Corrected Model	glycogen	24,666(b)	1	24,666	256,550	,000	,816	1,000
	activity	48623,913(c)	1	48623,913	83,877	,000	,591	1,000
	protein	,327(d)	1	,327	,198	,658	,003	,072
Intercept	glycogen	243,090	1	243,090	2528,401	,000	,978	1,000
	activity	502106,276	1	502106,276	866,142	,000	,937	1,000
	protein	709,534	1	709,534	430,118	,000	,881	1,000
genotype	glycogen	24,666	1	24,666	256,550	,000	,816	1,000
	activity	48623,913	1	48623,913	83,877	,000	,591	1,000
	protein	,327	1	,327	,198	,658	,003	,072
Error	glycogen	5,576	58	,096				
	activity	33622,865	58	579,705				
	protein	95,678	58	1,650				
Total	glycogen	273,332	60					
	activity	584353,055	60					
	protein	805,540	60					
Corrected Total	glycogen	30,242	59					
	activity	82246,779	59					
	protein	96,005	59					

a Computed using alpha = ,05

b R Squared = ,816 (Adjusted R Squared = ,812)

c R Squared = ,591 (Adjusted R Squared = ,584)

d R Squared = ,003 (Adjusted R Squared = -,014)

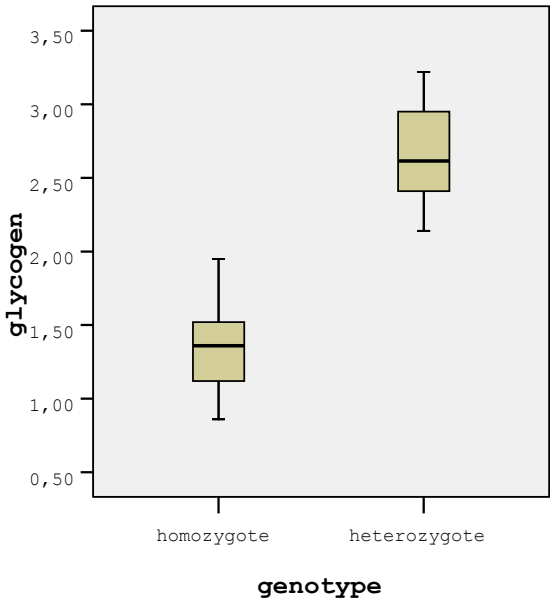


Figure 23. Glycogen content versus genotype chart at adult stage (mean=1.37, st. dev= 0.31 for homozygotes; mean=2.65 st.dev=0.31 for heterozygotes).

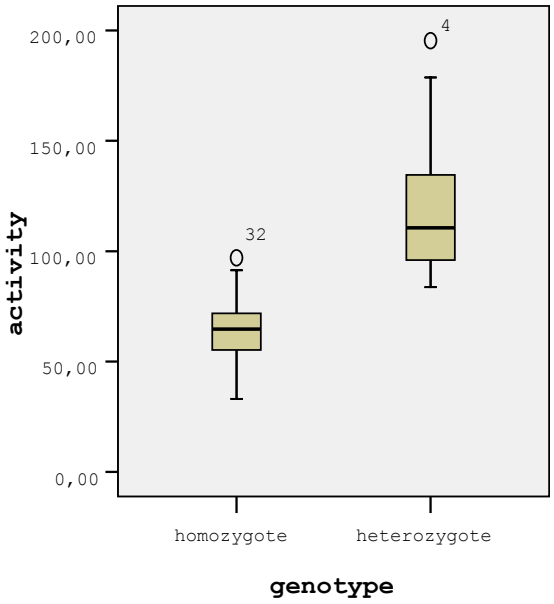


Figure 24. PGM activity versus genotype chart at adult stage (mean=63.01, st. dev= 14.36 for homozygotes; mean=119.95 st.dev=30.87 for heterozygotes).

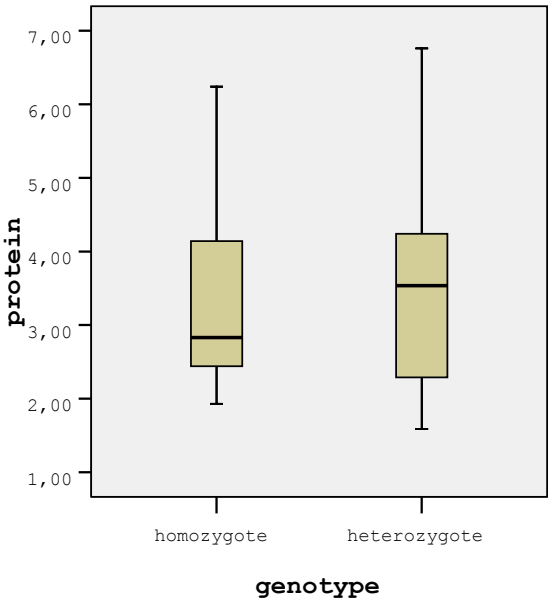


Figure 25. Protein content versus genotype chart adult stage (mean=3.37, st. dev= 1.25 for homozygotes; mean=3.51 st.dev=1.32 for heterozygotes).

3.2.2. Pupal Stage

3.2.2.1. Analysis of Protein Content

Protein content measurements were performed to standardize PGM enzyme activity and glycogen content measurements so that these two types of data become comparable independent of individual weight differences. Absorbance readings measured at 570 nm and protein concentrations of the samples determined from their corresponding standard curves. In order to compare three subspecies in terms of protein content, 20 individuals from each of the subspecies were assayed as shown in Tables 51, 52, and 53.

Table 51. Protein contents of *A. m. carnica* at pupal stage.

Winter			Summer		
		[Protein]			[Protein]
Sample	**Genotype**	**mg/mL**	**Sample**	**Genotype**	**mg/mL**
1	*Pgm 75/100*	3.98	1	*Pgm 75/100*	4.12
2	*Pgm 75/100*	4.52	2	*Pgm 75/75*	4.54
3	*Pgm 75/100*	4.07	3	*Pgm 75/75*	4.64
4	*Pgm 75/75*	5.61	4	*Pgm 75/75*	3.73
5	*Pgm 75/100*	3.45	5	*Pgm 75/100*	3.22
6	*Pgm 75/100*	3.14	6	*Pgm 75/75*	4.15
7	*Pgm 75/75*	6.73	7	*Pgm 75/75*	5.87
8	*Pgm 75/100*	4.35	8	*Pgm 75/75*	4.27
9	*Pgm 75/100*	4.05	9	*Pgm 75/75*	4.90
10	*Pgm 75/100*	6.93	10	*Pgm 75/75*	5.09
	Mean=4.68 SD=1.31			**Mean=4.45 SD=0.74**	

Table 52. Protein contents of *A . m. caucasica* at pupal stage.

Winter			Summer		
		[Protein]			[Protein]
Sample	**Genotype**	**mg/mL**	**Sample**	**Genotype**	**mg/mL**
1	*Pgm 75/75*	6.60	1	*Pgm 75/75*	4.97
2	*Pgm 75/100*	3.12	2	*Pgm 75/100*	3.61
3	*Pgm 75/100*	2.55	3	*Pgm 75/75*	5.25
4	*Pgm 75/100*	6.15	4	*Pgm 75/75*	5.44
5	*Pgm 75/100*	5.18	5	*Pgm 75/75*	5.86
6	*Pgm 75/100*	4.78	6	*Pgm 75/75*	4.88
7	*Pgm 75/100*	4.56	7	*Pgm 75/75*	3.74
8	*Pgm 75/75*	5.76	8	*Pgm 75/100*	5.67
9	*Pgm 75/100*	4.12	9	*Pgm 75/75*	5.81
10	*Pgm 75/100*	4.88	10	*Pgm 75/75*	5.32
	Mean=4.77 SD=1.27			**Mean=5.06 SD=0.80**	

Table 53. Protein contents of *A. m. syriaca* at pupal stage.

Winter			Summer		
Sample	Genotype	[Protein] mg/mL	Sample	Genotype	[Protein] mg/mL
1	*Pgm 75/75*	8.08	1	*Pgm 75/75*	6.41
2	*Pgm 75/100*	3.95	2	*Pgm 75/75*	5.07
3	*Pgm 75/100*	4.99	3	*Pgm 75/75*	7.52
4	*Pgm 75/100*	9.60	4	*Pgm 75/75*	5.16
5	*Pgm 75/100*	5.54	5	*Pgm 75/75*	5.77
6	*Pgm 75/100*	4.74	6	*Pgm 75/75*	6.68
7	*Pgm 75/100*	3.62	7	*Pgm 75/100*	4.92
8	*Pgm 75/100*	4.49	8	*Pgm 75/75*	6.44
9	*Pgm 75/100*	6.30	9	*Pgm 75/75*	6.73
10	*Pgm 75/100*	4.73	10	*Pgm 75/75*	7.08
	Mean=5.60 SD=1.90			**Mean=6.18 SD=0.90**	

3.2.2.2. Analysis of Glycogen Content

Absorbance readings measured at 450 nm and glycogen concentrations of the samples determined from their corresponding standard curves. In order to compare three subspecies in terms of glycogen content, 20 individuals from each of the subspecies were assayed as shown in Tables 54, 55, and 56.

Table 54. Glycogen contents of *A .m. carnica* at pupal stage.

Sample	Genotype	[Glycogen] mg/mg protein	Sample	Genotype	[Glycogen] mg/mg protein
1	*Pgm 75/100*	1.48	1	*Pgm 75/75*	1.56
2	*Pgm 75/100*	1.74	2	*Pgm 75/100*	1.17
3	*Pgm 75/75*	2.50	3	*Pgm 75/100*	1.27
4	*Pgm 75/100*	0.86	4	*Pgm 75/100*	1.24
5	*Pgm 75/100*	3.44	5	*Pgm 75/75*	1.73
6	*Pgm 75/100*	3.41	6	*Pgm 75/100*	1.03
7	*Pgm 75/75*	1.55	7	*Pgm 75/100*	1.13
8	*Pgm 75/100*	1.84	8	*Pgm 75/100*	1.44
9	*Pgm 75/100*	2.91	9	*Pgm 75/100*	0.79
10	*Pgm 75/75*	1.14	10	*Pgm 75/100*	1.26
	Mean= 2.09 SD= 0.92			**Mean= 1.26 SD= 0.27**	

Table 55. Glycogen contents of *A. m. caucasica* at pupal stage.

Sample	Genotype	[Glycogen] mg/mg protein	Sample	Genotype	[Glycogen] mg/mg protein
1	*Pgm 75/75*	0.88	1	*Pgm 75/75*	0.98
2	*Pgm 75/100*	1.95	2	*Pgm 75/100*	2.24
3	*Pgm 75/100*	2.14	3	*Pgm 75/75*	1.47
4	*Pgm 75/100*	3.03	4	*Pgm 75/75*	1.19
5	*Pgm 75/100*	2.99	5	*Pgm 75/75*	1.22
6	*Pgm 75/100*	2.49	6	*Pgm 75/75*	1.08
7	*Pgm 75/100*	1.34	7	*Pgm 75/75*	1.23
8	*Pgm 75/75*	1.09	8	*Pgm 75/100*	1.97
9	*Pgm 75/100*	1.57	9	*Pgm 75/75*	1.12
10	*Pgm 75/100*	1.63	10	*Pgm 75/75*	1.40
	Mean=1.91 SD=0.75			**Mean=1.39 SD=0.41**	

Table 56. Glycogen contents of *A. m. syriaca* at pupal stage.

Sample	Winter Genotype	[Glycogen] mg/mg protein	Sample	Summer Genotype	[Glycogen] mg/mg protein
1	*Pgm 75/75*	1.36	1	*Pgm 7575*	1.31
2	*Pgm 75/100*	2.75	2	*Pgm 75/75*	0.85
3	*Pgm 75/100*	2.35	3	*Pgm 75/75*	0.96
4	*Pgm 75/100*	1.97	4	*Pgm 75/75*	1.45
5	*Pgm 75/100*	1.62	5	*Pgm 75/75*	1.36
6	*Pgm 75/100*	1.74	6	*Pgm 75/75*	2.37
7	*Pgm 75/100*	3.14	7	*Pgm 75/100*	2.31
8	*Pgm 75/100*	2.68	8	*Pgm 75/75*	0.54
9	*Pgm 75/100*	1.77	9	*Pgm 75/75*	0.78
10	*Pgm 75/100*	1.85	10	*Pgm 75/75*	1.39
	Mean=2.12= SD=0.58			**Mean=1.23 SD=0.49**	

3.2.2.3. Analysis of PGM Activity

Absorbance readings measured at 340 nm and enzyme activities of the samples determined according to the formula:

µmol of NADP reduced/min/ml = (ΔA-340 nm/min) X (0.6 ml assay volume/6.2) X (1/0.033 ml extract volume) X (dilution factor).

Specific activities were standardized by their corresponding protein amounts. In order to compare three subspecies in terms of enzyme activity, 20 individuals from each of the subspecies were assayed as shown in Tables 57, 58, and 59.

Table 57. PGM activities of *A. m. carnica* at pupal stage.

Sample	Winter Genotype	PGM activity mUnits/mg protein	Sample	Summer Genotype	PGM activity mUnits/mg protein
1	*Pgm 75/100*	23.08	11	*Pgm 75/100*	24.73
2	*Pgm 75/100*	22.55	12	*Pgm 75/75*	11.37
3	*Pgm 75/100*	23.80	13	*Pgm 75/75*	16.75
4	*Pgm 75/75*	15.20	14	*Pgm 75/75*	17.62
5	*Pgm 75/100*	30.98	15	*Pgm 75/100*	34.75
6	*Pgm 75/100*	31.91	16	*Pgm 75/75*	18.34
7	*Pgm 75/75*	13.64	17	*Pgm 75/75*	15.20
8	*Pgm 75/100*	23.80	18	*Pgm 75/75*	18.07
9	*Pgm 75/100*	22.26	19	*Pgm 75/75*	14.53
10	*Pgm 75/75*	12.77	20	*Pgm 75/75*	15.33
	Mean=22.00 SD=6.57			**Mean=18.69 SD=6.61**	

Table 58. PGM activities of *A. m. caucasica* at pupal stage.

Sample	Winter Genotype	PGM activity mUnits/mg protein	Sample	Summer Genotype	PGM activity mUnits/mg protein
1	*Pgm 75/75*	15.95	11	*Pgm 75/75*	23.86
2	*Pgm 75/100*	40.15	12	*Pgm 75/100*	37.92
3	*Pgm 75/100*	48.46	13	*Pgm 75/75*	29.89
4	*Pgm 75/100*	41.00	14	*Pgm 75/75*	25.48
5	*Pgm 75/100*	32.92	15	*Pgm 75/75*	26.22
6	*Pgm 75/100*	34.47	16	*Pgm 75/75*	18.42
7	*Pgm 75/100*	35.29	17	*Pgm 75/75*	15.56
8	*Pgm 75/75*	17.40	18	*Pgm 75/100*	41.03
9	*Pgm 75/100*	35.27	19	*Pgm 75/75*	26.15
10	*Pgm 75/100*	36.37	20	*Pgm 75/75*	28.21
	Mean=33.73 SD=10.04			**Mean=27.27 SD=7.77**	

Table 59. PGM activities of *A. m. syriaca* at pupal stage.

Sample	Winter Genotype	PGM activity mUnits/mg protein	Sample	Summer Genotype	PGM activity mUnits/mg protein
1	*Pgm 75/75*	14.46	11	*Pgm 75/75*	17.20
2	*Pgm 75/100*	27.90	12	*Pgm 75/75*	18.62
3	*Pgm 75/100*	31.13	13	*Pgm 75/75*	22.21
4	*Pgm 75/100*	32.43	14	*Pgm 75/75*	23.63
5	*Pgm 75/100*	27.92	15	*Pgm 75/75*	23.73
6	*Pgm 75/100*	30.64	16	*Pgm 75/75*	20.76
7	*Pgm 75/100*	38.66	17	*Pgm 75/100*	32.58
8	*Pgm 75/100*	33.26	18	*Pgm 75/75*	20.49
9	*Pgm 75/100*	28.75	19	*Pgm 75/75*	27.12
10	*Pgm 75/100*	30.43	20	*Pgm 75/75*	16.52
	Mean=29.56 SD=6.18			**Mean=21.29 SD=4.77**	

3.2.2.4. Pearson Correlation for Biochemical Data at Pupal Stage

Table 60. Pearson product-moment correlation of biochemical data of *A. m. carnica* subspecies at pupal stage.

		glycogen	activity	protein
glycogen	Pearson Correlation	1	,733(**)	-,538(*)
	Sig. (2-tailed)		,000	,014
	N	20	20	20
activity	Pearson Correlation	,733(**)	1	-,763(**)
	Sig. (2-tailed)	,000		,000
	N	20	20	20
protein	Pearson Correlation	-,538(*)	-,763(**)	1
	Sig. (2-tailed)	,014	,000	
	N	20	20	20

** Correlation is significant at the 0.01 level (2-tailed).

* Correlation is significant at the 0.05 level (2-tailed).

As far as *A. m. carnica* subspecies of pupal stage is concerned, there is a positive strong correlation between glycogen content and PGM activity at the

0.01 significance level (r=0.733, p=0.00) as shown in Table 60. However, there is a negative strong correlation between protein content and Pgm activity (r= -0.763, p= 0.00). Also, there is a negative medium correlation between protein content and glycogen content (r= -0.538, p= 0.014).

Table 61. Pearson product-moment correlation of biochemical data of *A. m. caucasica* subspecies at pupal stage

		glycogen	activity	protein
glycogen	Pearson Correlation	1	,714(**)	-,210
	Sig. (2-tailed)		,000	,375
	N	20	20	20
activity	Pearson Correlation	,714(**)	1	-,453(*)
	Sig. (2-tailed)	,000		,045
	N	20	20	20
protein	Pearson Correlation	-,210	-,453(*)	1
	Sig. (2-tailed)	,375	,045	
	N	20	20	20

** Correlation is significant at the 0.01 level (2-tailed).

* Correlation is significant at the 0.05 level (2-tailed).

As far *as A. m. caucasica* subspecies of pupal stage is concerned, there is a positive strong correlation between glycogen content and PGM activity at the 0.01 significance level (r=0.714, p=0.00) as shown in Table 61. However, there is a negative medium correlation between protein content and PGM activity but this correlation is not statistically significant (r= -0.453, p= 0.045). Also, there is a negative weak correlation between protein content and glycogen content but this correlation is not statistically significant (r= -0.210, p= 0.375).

As far as *A. m. syriaca* subspecies of pupal stage is concerned, there is a positive strong correlation between glycogen content and PGM activity at the 0.01 significance level (r=0.821, p=0.00) as shown in Table 62. However, there is a negative medium correlation between protein content and PGM activity (r= -0.488, p= 0.029). Also, there is a negative medium correlation between protein content and glycogen content (r= -0.529, p= 0.016).

Table 62. Pearson product-moment correlation of biochemical data of *A. m. syriaca* subspecies at adult stage

		glycogen	activity	protein
glycogen	Pearson Correlation	1	,821(**)	-,529(*)
	Sig. (2-tailed)		,000	,016
	N	20	20	20
activity	Pearson Correlation	,821(**)	1	-,488(*)
	Sig. (2-tailed)	,000		,029
	N	20	20	20
protein	Pearson Correlation	-,529(*)	-,488(*)	1
	Sig. (2-tailed)	,016	,029	
	N	20	20	20

** Correlation is significant at the 0.01 level (2-tailed).

* Correlation is significant at the 0.05 level (2-tailed).

3.2.2.5. MANOVA Results for Biochemical Data at Pupal Stage

3.2.2.5.1. Subspecies vs. Biochemical Data

A one-way between groups multivariate analysis of variance (MANOVA) was performed to investigate subspecies differences in PGM activity, glycogen and protein content data for pupa. Three independet variables were used: glycogen content, protein content and PGM activity. The independent variable was subspecies of *A. mellifera* L. Preliminary assumption testing was conducted to check for normality, linearity, univariate and multivariate outliers, homogeneity of variance-covariance matrices, and multicollinearity, with no serious violations noted.

There is a statistically significant difference between subspecies of *A. mellifera* on the combined dependent variables of pupal stage as shown in Table 63 (F=9.90 and p=0.00 for Wilks' Lambda=0.422, partial eta squared=0.351)

Table 63. Multivariate Tests(d) for subspecies at pupal stage.

Effect		Value	F	df	Sig.	Partial Eta Squared	Observed Power(a)
Intercept	Pillai's Trace	,985	1171,440(b)	3	,000	,985	1,000
	Wilks' Lambda	,015	1171,440(b)	3	,000	,985	1,000
	Hotelling's Trace	63,897	1171,440(b)	3	,000	,985	1,000
	Roy's Largest Root	63,897	1171,440(b)	3	,000	,985	1,000
subsp	Pillai's Trace	,666	9,324	6	,000	,333	1,000
	Wilks' Lambda	,422	9,900(b)	6	,000	,351	1,000
	Hotelling's Trace	1,163	10,470	6	,000	,368	1,000
	Roy's Largest Root	,942	17,587(c)	3	,000	,485	1,000

a Computed using alpha = ,05

b Exact statistic

c The statistic is an upper bound on F that yields a lower bound on the significance level.

d Design: Intercept+subsp

Table 64. Levene's Test of Equality of Error Variances(a) for subspecies at pupal stage.

	F	df1	df2	Sig.
glycogen	,099	2	57	,906
activity	1,965	2	57	,149
protein	1,981	2	57	,147

Tests the null hypothesis that the error variance of the dependent variable is equal across groups.

a Design: Intercept+subsp

Table 64 indicates that variances of glycogen content, protein content and enzyme activity data are equal among subspecies at pupal stage (F= 0,099 and p=0.906; F=1.981, p=0.147; F=1,965 and p=1.147 respectively).

When the results for the dependent variables were considered seperately, PGM activity and protein content reach statistically significant difference among subspecies at pupal stage (F= 8.687, p=0.001, partial eta squared= 0.234; F=6.502, p=0.003, partial eta squared=0.186). This means that 23.4 per cent of the variance in enzyme activity data and 18.6 per cent of the variance in

enzyme activity data are explained by subspecies of *A. mellifera* as shown in Table 65.

Table 65. Tests of Between-Subjects Effects for subspecies at pupal stage.

Source	Dependent Variable	Type III Sum of Squares	df	Mean Square	F	Sig.	Partial Eta Squared	Observed Power(a)
Corrected Model	glycogen	,009(b)	2	,004	,009	,991	,000	,051
	activity	1031,646(c)	2	515,823	8,687	,001	,234	,962
	protein	18,843(d)	2	9,422	6,502	,003	,186	,892
Intercept	glycogen	166,833	1	166,833	330,4	,000	,853	1,000
	activity	38777,70	1	38777,70	653,0	,000	,920	1,000
	protein	1575,220	1	1575,220	1087,	,000	,950	1,000
subsp	glycogen	,009	2	,004	,009	,991	,000	,051
	activity	1031,646	2	515,823	8,687	,001	,234	,962
	protein	18,843	2	9,422	6,502	,003	,186	,892
Error	glycogen	28,775	57	,505				
	activity	3384,668	57	59,380				
	protein	82,594	57	1,449				
Total	glycogen	195,618	60					
	activity	43194,01	60					
	protein	1676,657	60					
Corrected Total	glycogen	28,784	59					
	activity	4416,314	59					
	protein	101,437	59					

a Computed using alpha = ,05

b R Squared = ,000 (Adjusted R Squared = -,035)

c R Squared = ,234 (Adjusted R Squared = ,207)

d R Squared = ,186 (Adjusted R Squared = ,157)

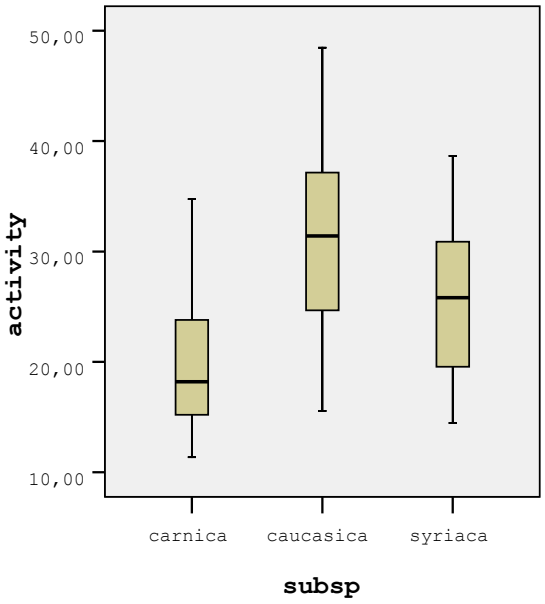


Figure 26. PGM activity versus subspecies of *A. mellifera* chart at pupal stage (mean=20.34, st. dev= 6.63 for *A. m. carnica* ; mean=30.50, st.dev=9.32 for *A. m. caucasica* ; mean=25.42 st.dev=6.84 for *A. m. syriaca*)

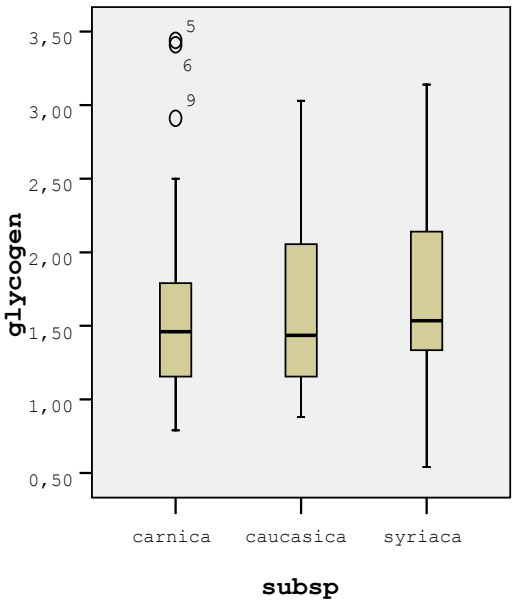


Figure 27. Glycogen content versus subspecies of *A. mellifera* chart at pupal stage (mean=1.67, st. dev= 0.68 for *A. m. carnica* ; mean=1.65, st.dev=0.65 for *A. m. caucasica* ; mean=1.68 st.dev=0.69 for *A. m. syriaca*)

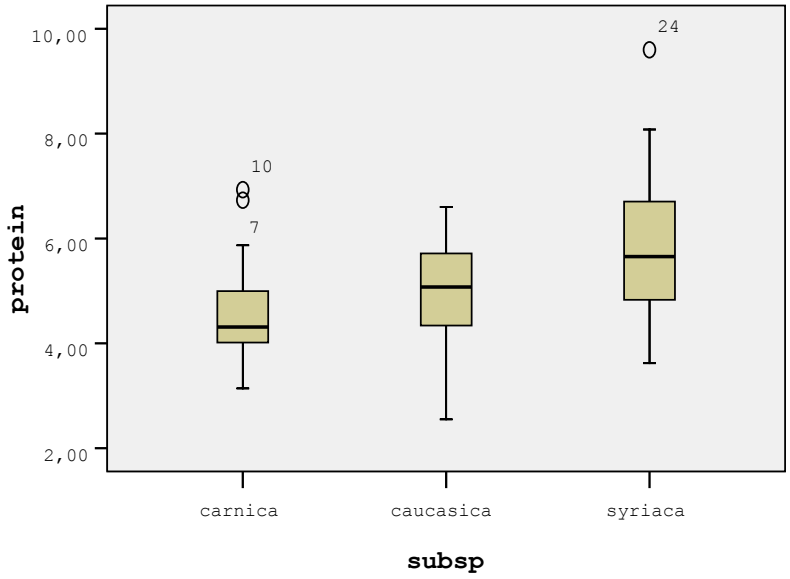


Figure 28. Protein content versus subspecies of *A. mellifera* chart at pupal stage (mean=4.57, st. dev= 1.04 for *A. m. carnica* ; mean=4.91, st.dev=1.04 for *A. m. caucasica* ; mean=5.89 st.dev=1.48 for *A. m. syriaca*)

3.2.2.5.2. Season vs. Biochemical Data

A one-way between groups multivariate analysis of variance (MANOVA) was performed to investigate seasonal differences in biochemical data at pupal stage. Three independet variables were used: glycogen content, protein content and PGM activity. The independent variable was season. Preliminary assumption testing was conducted to check for normality, linearity, univariate and multivariate outliers, homogeneity of variance-covariance matrices, and multicollinearity, with no serious violations noted.

There is a statistically significant difference between seasons on the combined dependent variables of pupal stage as shown in Table 66 (F=8.429, p=0.00, Wilks' Lambda=0.689, partial eta squared=0.311).

Table 66. Multivariate Tests (d) for seasons at pupal stage.

Effect		Value	F	df	Sig.	Partial Eta Squared	Observed Power(a)
Intercept	Pillai's Trace	,980	899,700(b)	3	,000	,980	1,000
	Wilks' Lambda	,020	899,700(b)	3	,000	,980	1,000
	Hotelling's Trace	48,198	899,700(b)	3	,000	,980	1,000
	Roy's Largest Root	48,198	899,700(b)	3	,000	,980	1,000
season	Pillai's Trace	,311	8,429(b)	3	,000	,311	,990
	Wilks' Lambda	,689	8,429(b)	3	,000	,311	,990
	Hotelling's Trace	,452	8,429(b)	3	,000	,311	,990
	Roy's Largest Root	,452	8,429(b)	3	,000	,311	,990

a Computed using alpha = ,05

b Exact statistic

c Design: Intercept+season

Table 67. Levene's Test of Equality of Error Variances (a)for seasons at pupal stage.

	F	df1	df2	Sig.
glycogen	15,991	1	58	,000
activity	1,459	1	58	,232
protein	2,082	1	58	,154

Tests the null hypothesis that the error variance of the dependent variable is equal across groups.

a Design: Intercept+season

Table 67 indicates that variances of glycogen content data are not equal among subspecies (F= 15,991 and p=0.00) whereas variances of PGM activity and protein content data are equal among subspecies at pupal stage (F=1.459, p=0.232; F=2.082, p=0.154).

Table 68. Tests of Between-Subjects Effects for seasons at pupal stage.

Source	Dependent Variable	Type III Sum of Squares	df	Mean Square	F	Sig.	Partial Eta Squared	Observed Power(a)
Corrected Model	glycogen	8,340(b)	1	8,340	23,662	,000	,290	,998
	activity	542,162(c)	1	542,162	8,117	,006	,123	,800
	protein	,659(d)	1	,659	,380	,540	,007	,093
Intercept	glycogen	166,833	1	166,833	473,313	,000	,891	1,000
	activity	38777,702	1	38777,70	580,542	,000	,909	1,000
	protein	1575,220	1	1575,220	906,574	,000	,940	1,000
season	glycogen	8,340	1	8,340	23,662	,000	,290	,998
	activity	542,162	1	542,162	8,117	,006	,123	,800
	protein	,659	1	,659	,380	,540	,007	,093
Error	glycogen	20,444	58	,352				
	activity	3874,152	58	66,796				
	protein	100,778	58	1,738				
Total	glycogen	195,618	60					
	activity	43194,016	60					
	protein	1676,657	60					
Corrected Total	glycogen	28,784	59					
	activity	4416,314	59					
	protein	101,437	59					

a Computed using alpha = ,05

b R Squared = ,290 (Adjusted R Squared = ,278)

c R Squared = ,123 (Adjusted R Squared = ,108)

d R Squared = ,007 (Adjusted R Squared = -,011)

When the results for the dependent variables were considered seperately, PGM activity and glycogen content data exhibit statistically significant difference between seasons at pupal stage (F= 8.12, p=0.006, partial eta square=0.123; F=23.66, p=0.00, partial eta square=0.29 respectively). This means that 12.3 per cent of the variance in enzyme activity data and 29.0 per cent of the variance in glycogen content data are explained by seasonal differentiation as shown in Table 68.

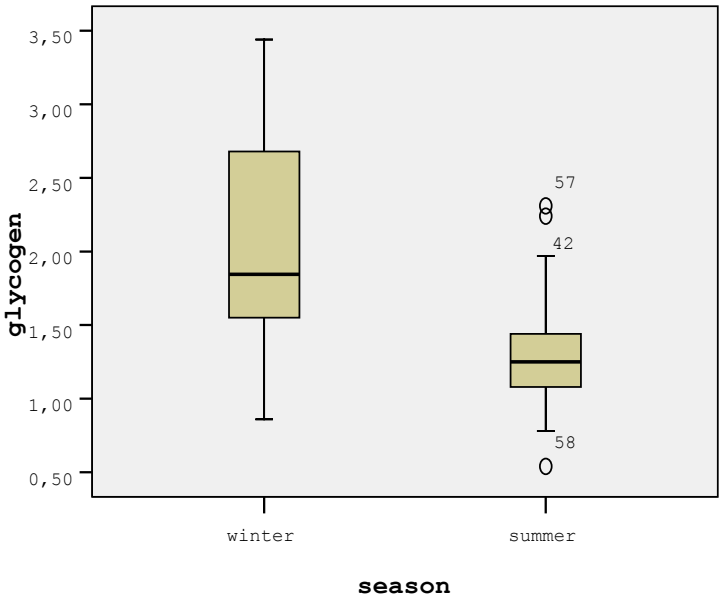


Figure 29. Glycogen content versus season chart at pupal stage (mean=2.04, st. dev= 0.72 for winter; mean=1.29 st.dev=0.39 for summer).

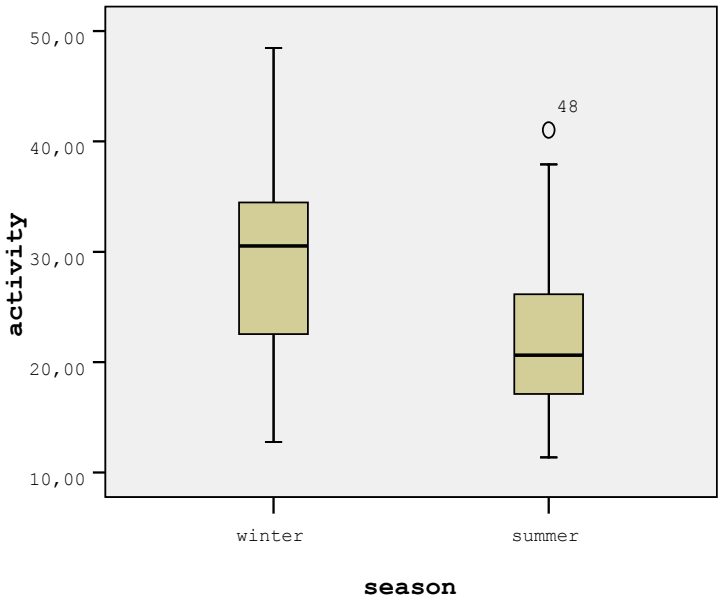


Figure 30. PGM activity versus season chart at pupal stage (mean=28.43, st. dev= 8.99 for summer; mean=33.41 st.dev=7.26 for winter).

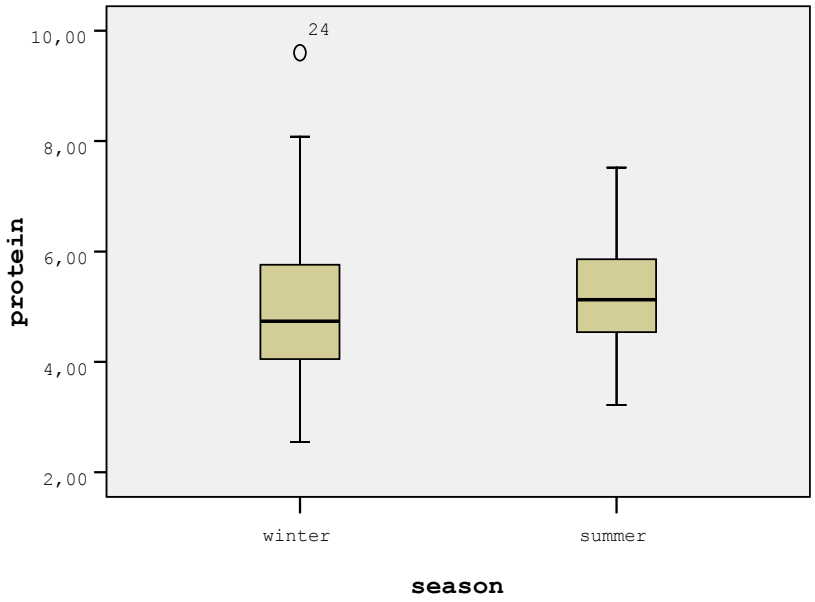


Figure 31. Protein content versus season chart at pupal stage (mean=5.02, st. dev= 1.53 for winter; mean=5.23 st.dev=1.07 for summer).

3.2.2.5.3. Genotype vs. Biochemical Data

A one-way between groups multivariate analysis of variance (MANOVA) was performed to investigate genotypic differences in PGM activity, glycogen and protein content data at pupal stage. Three independet variables were used: glycogen content, protein content and PGM activity. The independent variable was genotype. Preliminary assumption testing was conducted to check for normality, linearity, univariate and multivariate outliers, homogeneity of variance-covariance matrices, and multicollinearity, with no serious violations noted.

There is a statistically significant difference between genotypes of *Pgm* allozymes on the combined dependent variables at pupal stage as shown in Table 69 (F=47.528, p=0.00, Wilks' Lambda=0.282, partial eta squared=0.718).

Table 69. Multivariate Tests (d) for genotypes at pupal stage.

Effect		Value	F	df	Sig.	Partial Eta Squared	Observed Power(a)
Intercept	Pillai's Trace	,983	1080,590(b)	3	,000	,983	1,000
	Wilks' Lambda	,017	1080,590(b)	3	,000	,983	1,000
	Hotelling's Trace	57,889	1080,590(b)	3	,000	,983	1,000
	Roy's Largest Root	57,889	1080,590(b)	3	,000	,983	1,000
genotype	Pillai's Trace	,718	47,528(b)	3	,000	,718	1,000
	Wilks' Lambda	,282	47,528(b)	3	,000	,718	1,000
	Hotelling's Trace	2,546	47,528(b)	3	,000	,718	1,000
	Roy's Largest Root	2,546	47,528(b)	3	,000	,718	1,000

a Computed using alpha = ,05

b Exact statistic

c Design: Intercept+genotype

Table 70. Levene's Test of Equality of Error Variances (a) for genotypes at pupal stage

	F	df1	df2	Sig.
glycogen	27,793	1	58	,000
activity	1,300	1	58	,259
protein	,000	1	58	,998

Tests the null hypothesis that the error variance of the dependent variable is equal across groups.

a Design: Intercept+genotype

Table 70 indicates that variances of glycogen content data are not equal among genotypes (F= 27,793 and p=0.00) whereas variances of PGM activity and protein content data are equal among genotypes at pupal stage (F=1.30, p=0.259; F=0.00, p=0.998 respectively).

Table 71. Tests of Between-Subjects Effects for genotypes at pupal stage.

Source	Dependent Variable	Type III Sum of Squares	df	Mean Square	F	Sig.	Partial Eta Squared	Observed Power(a)
Corrected Model	glycogen	16,624(b)	1	16,624	79,295	,000	,578	1,000
	activity	2595,205(c)	1	2595,205	82,654	,000	,588	1,000
	protein	17,496(d)	1	17,496	12,089	,001	,172	,928
Intercept	glycogen	170,175	1	170,175	811,703	,000	,933	1,000
	activity	39405,911	1	39405,911	1255,027	,000	,956	1,000
	protein	1562,428	1	1562,428	1079,577	,000	,949	1,000
genotype	glycogen	16,624	1	16,624	79,295	,000	,578	1,000
	activity	2595,205	1	2595,205	82,654	,000	,588	1,000
	protein	17,496	1	17,496	12,089	,001	,172	,928
Error	glycogen	12,160	58	,210				
	activity	1821,110	58	31,398				
	protein	83,941	58	1,447				
Total	glycogen	195,618	60					
	activity	43194,016	60					
	protein	1676,657	60					
Corrected Total	glycogen	28,784	59					
	activity	4416,314	59					
	protein	101,437	59					

a Computed using alpha = ,05

b R Squared = ,578 (Adjusted R Squared = ,570)

c R Squared = ,588 (Adjusted R Squared = ,581)

d R Squared = ,172 (Adjusted R Squared = ,158)

When the results for the dependent variables were considered seperately; glycogen content, PGM activity and protein content data exhibit statistically significant difference among genotypes at pupal stage (F= 79.30, p=0.00, partial eta square=0.578; F=82.654, p=0.00, partial eta square= 0.588; F=12,09, p=0.001, partial eta square=0.172). This means that 57.8 per cent of the variance in glycogen content data and 58.8 per cent of the variance in enzyme activity data and 17.2 per cent of the variance in protein content data are explained by seasonal differentiation in pupal stage as shown in Table 71.

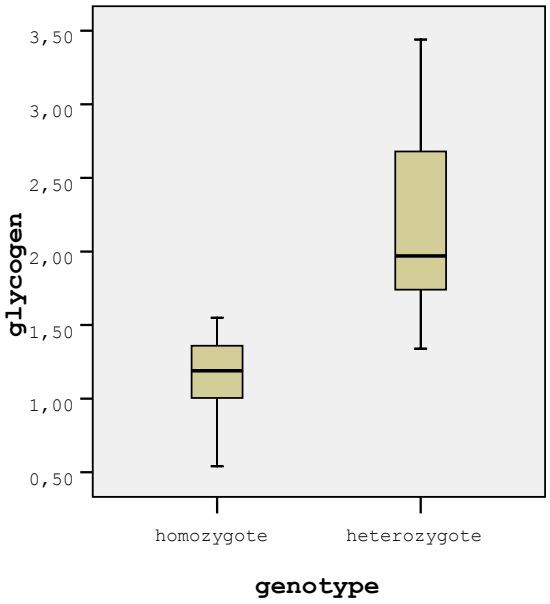


Figure 32. Glycogen content versus genotype chart at pupal stage (mean=1.56, st. dev= 0.24 for homozygotes; mean=2.21 st.dev=0.61 for heterozygotes).

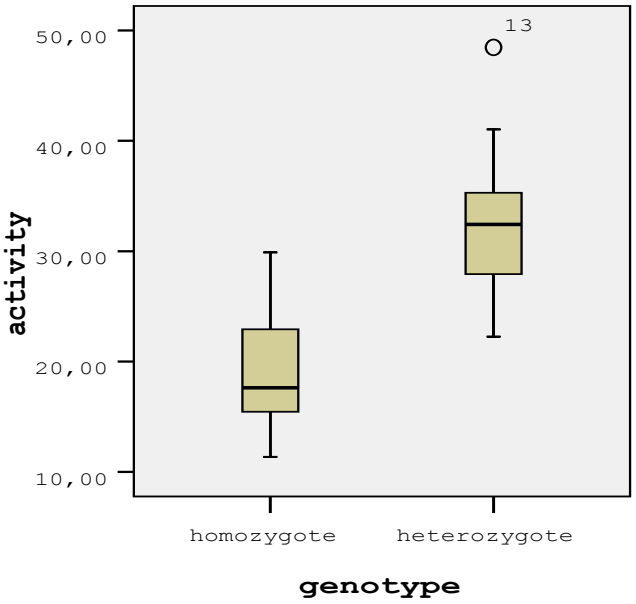


Figure 33. PGM activity versus genotype chart at pupal stage (mean=19.06, st. dev= 4.78 for homozygotes; mean=32.22 st.dev=6.37 for heterozygotes).

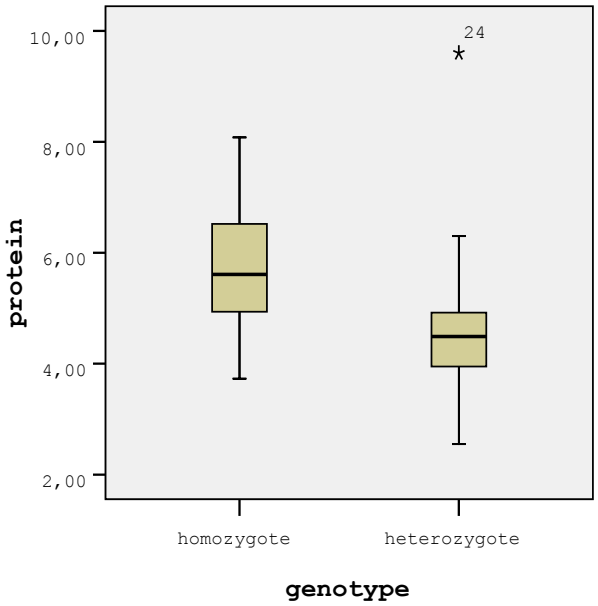


Figure 34. Protein content versus genotype chart at pupal stage (mean=5.65, st. dev= 1.08 for homozygotes; mean=4.57 st.dev=1.32 for heterozygotes).

3.2.3. Larval Stage

3.2.3.1. Analysis of Protein Content

Protein content measurements were performed to standardize PGM enzyme activity and glycogen content measurements so that these two types of data become comparable independent of individual weight differences. Absorbance readings measured at 570 nm and protein concentrations of the samples determined from their corresponding standard curves. In order to compare three subspecies in terms of protein content, 20 individuals from each of the subspecies were assayed as shown in Tables 72, 73, and 74.

Table 72. Protein contents of *A. m. carnica* at larval stage.

	Winter			Summer	
		[Protein]			[Protein]
Sample	**Genotype**	**mg/mL**	**Sample**	**Genotype**	**mg/mL**
1	*Pgm 75/100*	1.07	1	*Pgm 75/100*	0.90
2	*Pgm 75/100*	1.87	2	*Pgm 75/75*	1.08
3	*Pgm 75/100*	2.26	3	*Pgm 75/75*	0.73
4	*Pgm 75/100*	1.25	4	*Pgm 75/75*	0.87
5	*Pgm 75/100*	2.11	5	*Pgm 75/75*	0.90
6	*Pgm 75/100*	2.31	6	*Pgm 75/75*	0.76
7	*Pgm 75/75*	2.05	7	*Pgm 75/75*	0.79
8	*Pgm 75/100*	2.43	8	*Pgm 75/75*	0.56
9	*Pgm 75/75*	1.94	9	*Pgm 75/75*	0.80
10	*Pgm 75/100*	2.01	10	*Pgm 75/75*	0.82
	Mean=1.93 SD=0.44			**Mean=0.82 SD=0.13**	

Table 73. Protein contents of *A. m. caucasica* at larval stage.

	Winter			Summer	
		[Protein]			[Protein]
Sample	**Genotype**	**mg/mL**	**Sample**	**Genotype**	**mg/mL**
1	*Pgm 75/100*	1.28	1	*Pgm 75/75*	1.14
2	*Pgm 75/100*	1.43	2	*Pgm 75/75*	0.58
3	*Pgm 75/100*	0.75	3	*Pgm 75/100*	1.07
4	*Pgm 75/100*	1.64	4	*Pgm 75/75*	1.00
5	*Pgm 75/75*	2.11	5	*Pgm 75/75*	1.31
6	*Pgm 75/75*	1.85	6	*Pgm 75/75*	1.48
7	*Pgm 75/100*	1.03	7	*Pgm 75/75*	0.60
8	*Pgm 75/100*	0.81	8	*Pgm 75/75*	0.52
9	*Pgm 75/100*	0.88	9	*Pgm 75/75*	0.34
10	*Pgm 75/100*	1.20	10	*Pgm 75/100*	0.70
	Mean=1.30 SD=0.46			**Mean=0.87 SD=0.38**	

Table 74. Protein contents of *A. m. syriaca* at larval stage.

Sample	Genotype	[Protein] mg/mL	Sample	Genotype	[Protein] mg/mL
1	*Pgm 75/100*	1.29	1	*Pgm 75/75*	0.80
2	*Pgm 75/100*	1.18	2	*Pgm 75/100*	0.33
3	*Pgm 75/100*	1.68	3	*Pgm 75/75*	0.36
4	*Pgm 75/100*	1.02	4	*Pgm 75/75*	0.63
5	*Pgm 75/100*	1.18	5	*Pgm 75/75*	0.50
6	*Pgm 75/100*	1.42	6	*Pgm 75/75*	0.46
7	*Pgm 75/100*	0.96	7	*Pgm 75/75*	0.41
8	*Pgm 75/75*	0.74	8	*Pgm 75/100*	0.27
9	*Pgm 75/100*	0.79	9	*Pgm 75/75*	0.48
10	*Pgm 75/100*	1.92	10	*Pgm 75/75*	0.50
	Mean=1.22 SD=0.38			**Mean=0.47 SD=0.15**	

3.2.3.2. Analysis of Glycogen Content

Absorbance readings measured at 450 nm and glycogen concentrations of the samples determined from their corresponding standard curves. In order to compare three subspecies in terms of glycogen content, 20 individuals from each of the subspecies were assayed as shown in Tables 75, 76, and 77.

Table 75. Glycogen contents of *A. m. carnica* at larval stage.

Sample	Winter Genotype	[Glycogen] mg/mg protein	Sample	Summer Genotype	[Glycogen] mg/mg protein
1	*Pgm 75/100*	2.58	1	*Pgm 75100*	1.80
2	*Pgm 75/100*	1.26	2	*Pgm 75/75*	0.83
3	*Pgm 75/100*	1.47	3	*Pgm 75/75*	0.54
4	*Pgm 75/100*	1.59	4	*Pgm 75/75*	0.70
5	*Pgm 75/100*	1.13	5	*Pgm 75/75*	0.62
6	*Pgm 75/100*	1.25	6	*Pgm 75/75*	1.09
7	*Pgm 75/75*	0.68	7	*Pgm 75/75*	0.98
8	*Pgm 75/100*	2.30	8	*Pgm 75/75*	0.63
9	*Pgm 75/75*	0.76	9	*Pgm 75/75*	0.63
10	*Pgm 75/100*	1.62	10	*Pgm 75/75*	0.78
	Mean=1.46 SD=0.60			**Mean=0.86 SD=0.37**	

Table 76. Glycogen contents of *A. m. caucasica* at larval stage.

Sample	Winter Genotype	[Glycogen] mg/mg protein	Sample	Summer Genotype	[Glycogen] mg/mg protein
1	*Pgm 75/100*	1.21	1	*Pgm 75/75*	0.88
2	*Pgm 75/100*	1.09	2	*Pgm 75/75*	1.00
3	*Pgm 75/100*	2.46	3	*Pgm 75/100*	1.66
4	*Pgm 75/100*	2.32	4	*Pgm 75/75*	0.71
5	*Pgm 75/75*	0.88	5	*Pgm 75/75*	0.69
6	*Pgm 75/75*	0.97	6	*Pgm 75/75*	0.87
7	*Pgm 75/100*	1.69	7	*Pgm 75/75*	0.58
8	*Pgm 75/100*	2.20	8	*Pgm 75/75*	0.48
9	*Pgm 75/100*	1.57	9	*Pgm 75/75*	1.04
10	*Pgm 75/100*	2.57	10	*Pgm 75/100*	1.94
	Mean=1.70 SD=0.65			**Mean=0.98 SD=0.47**	

Table 77. Glycogen contents of *A. m. syriaca* at larval stage.

Sample	Winter Genotype	[Glycogen] mg/mg protein	Sample	Summer Genotype	[Glycogen] mg/mg protein
1	*Pgm 75/100*	1.20	1	*Pgm 7575*	0.88
2	*Pgm 75/100*	1.46	2	*Pgm 75/100*	2.66
3	*Pgm 75/100*	2.53	3	*Pgm 75/75*	1.10
4	*Pgm 75/100*	1.57	4	*Pgm 75/75*	0.63
5	*Pgm 75/100*	1.87	5	*Pgm 75/75*	0.62
6	*Pgm 75/100*	1.63	6	*Pgm 75/75*	1.08
7	*Pgm 75/100*	2.07	7	*Pgm 75/75*	0.56
8	*Pgm 75/75*	0.50	8	*Pgm 75/100*	0.86
9	*Pgm 75/100*	2.06	9	*Pgm 75/75*	0.58
10	*Pgm 75/100*	1.98	10	*Pgm 75/75*	0.70
	Mean=1.69 SD=0.56			**Mean=0.96 SD=0.63**	

3.2.3.3. Analysis of PGM Activity

Absorbance readings measured at 340 nm and enzyme activities of the samples determined according to the formula:

μmol of NADP reduced/min/ml = (ΔA-340 nm/min) X (0.6 ml assay volume/6.2) X (1/0.033 ml extract volume) X (dilution factor).

Specific activities were standardized by their corresponding protein amounts. In order to compare three subspecies in terms of enzyme activity, 20 individuals from each of the subspecies were assayed as shown in Tables 78, 79, and 80.

Table 78. PGM activities of *A. m. carnica* at larval stage.

Sample	Winter Genotype	PGM activity mUnits/mg protein	Sample	Summer Genotype	PGM activity mUnits/mg protein
1	*Pgm 75/100*	25.70	11	*Pgm 75/100*	18.34
2	*Pgm 75/100*	23.60	12	*Pgm 75/75*	14.48
3	*Pgm 75/100*	23.28	13	*Pgm 75/75*	7.31
4	*Pgm 75/100*	20.71	14	*Pgm 75/75*	10.24
5	*Pgm 75/100*	25.05	15	*Pgm 75/75*	15.18
6	*Pgm 75/100*	20.84	16	*Pgm 75/75*	14.14
7	*Pgm 75/75*	12.22	17	*Pgm 75/75*	16.32
8	*Pgm 75/100*	24.58	18	*Pgm 75/75*	7.18
9	*Pgm 75/75*	14.81	19	*Pgm 75/75*	15.20
10	*Pgm 75/100*	22.44	20	*Pgm 75/75*	7.15
	Mean=21.32 SD=4.47			**Mean=12.55 SD=4.20**	

Table 79. PGM activities of *A. m. caucasica* at larval stage.

Sample	Winter Genotype	PGM activity mUnits/mg protein	Sample	Summer Genotype	PGM activity mUnits/mg protein
1	*Pgm 75/100*	19.72	11	*Pgm 75/75*	14.33
2	*Pgm 75/100*	18.70	12	*Pgm 75/75*	11.17
3	*Pgm 75/100*	19.32	13	*Pgm 75/100*	18.02
4	*Pgm 75/100*	25.28	14	*Pgm 75/75*	15.13
5	*Pgm 75/75*	11.72	15	*Pgm 75/75*	13.29
6	*Pgm 75/75*	16.38	16	*Pgm 75/75*	7.92
7	*Pgm 75/100*	19.59	17	*Pgm 75/75*	10.57
8	*Pgm 75/100*	30.55	18	*Pgm 75/75*	14.75
9	*Pgm 75/100*	17.79	19	*Pgm 75/75*	15.81
10	*Pgm 75/100*	39.36	20	*Pgm 75/100*	29.07
	Mean=21.84 SD=7.95			**Mean=15.01 SD=5.73**	

Table 80. PGM activities of *A. m. syriaca* at larval stage.

Sample	Winter Genotype	PGM activity mUnits/mg protein	Sample	Summer Genotype	PGM activity mUnits/mg protein
1	*Pgm 75/100*	18.27	11	*Pgm 75/75*	16.30
2	*Pgm 75/100*	21.09	12	*Pgm 75/100*	23.08
3	*Pgm 75/100*	25.40	13	*Pgm 75/75*	13.78
4	*Pgm 75/100*	18.95	14	*Pgm 75/75*	13.31
5	*Pgm 75/100*	20.77	15	*Pgm 75/75*	13.86
6	*Pgm 75/100*	23.50	16	*Pgm 75/75*	12.27
7	*Pgm 75/100*	28.12	17	*Pgm 75/75*	15.48
8	*Pgm 75/75*	16.25	18	*Pgm 75/100*	22.51
9	*Pgm 75/100*	24.65	19	*Pgm 75/75*	15.48
10	*Pgm 75/100*	27.05	20	*Pgm 75/75*	15.00
	Mean=22.40 SD=3.97			**Mean=16.11 SD=3.72**	

3.2.3.4. Pearson Correlation of Biochemical Data at Larval Stage

Table 81. Pearson product-moment correlation of biochemical data of *A. m. carnica* subspecies at larval stage.

		glycogen	activity	protein
glycogen	Pearson Correlation	1	,781(**)	,366
	Sig. (2-tailed)		,000	,113
	N	20	20	20
activity	Pearson Correlation	,781(**)	1	,648(**)
	Sig. (2-tailed)	,000		,002
	N	20	20	20
protein	Pearson Correlation	,366	,648(**)	1
	Sig. (2-tailed)	,113	,002	
	N	20	20	20

** Correlation is significant at the 0.01 level (2-tailed).

As far as *A. m. carni*ca subspecies of larval stage is concerned, there is a positive strong correlation between glycogen content and PGM activity at the

0.01 significance level (r=0.781, p=0.00) as shown in Table 81. However, there is a positive strong correlation between protein content and PGM activity (r= 0.648, p= 0.002). Also, there is a positive medium correlation between protein content and glycogen content but this correlation is not statistically significant (r= 0.336, p= 0.113).

Table 82. Pearson product-moment correlation of biochemical data of *A. m. caucasica* subspecies at larval stage.

		glycogen	activity	protein
glycogen	Pearson Correlation	1	,823(**)	-,026
	Sig. (2-tailed)		,000	,915
	N	20	20	20
activity	Pearson Correlation	,823(**)	1	-,049
	Sig. (2-tailed)	,000		,837
	N	20	20	20
protein	Pearson Correlation	-,026	-,049	1
	Sig. (2-tailed)	,915	,837	
	N	20	20	20

** Correlation is significant at the 0.01 level (2-tailed).

As far as *A. m. caucasic*a subspecies of larval stage is concerned, there is a positive strong correlation between glycogen content and PGM activity at the 0.01 significance level (r=0.823, p=0.00) as shown in Table 82. However, there is a negative weak correlation between protein content and PGM activity but this correlation is not statistically significant (r= -0.049, p= 0.872). Also, there is a negative weak correlation between protein content and glycogen content but this correlation is not statistically significant (r= -0.026, p= 0.915).

As far as *A. m. syriaca* subspecies of larval stage is concerned, there is a positive strong correlation between glycogen content and PGM activity at the 0.01 significance level (r=0.809, p=0.00) as shown in Table 83. However, there is a positive strong correlation between protein content and PGM activity (r= 0.604, p= 0.005). Also, there is a positive strong correlation between protein

content and glycogen content but this correlation is not statistically significant (r= 0.526, p= 0.017).

Table 83. Pearson product-moment correlation of biochemical data of *A. m. syriaca* subspecies at larval stage.

		glycogen	activity	protein
glycogen	Pearson Correlation	1	,809(**)	,526(*)
	Sig. (2-tailed)		,000	,017
	N	20	20	20
activity	Pearson Correlation	,809(**)	1	,604(**)
	Sig. (2-tailed)	,000		,005
	N	20	20	20
protein	Pearson Correlation	,526(*)	,604(**)	1
	Sig. (2-tailed)	,017	,005	
	N	20	20	20

** Correlation is significant at the 0.01 level (2-tailed).

* Correlation is significant at the 0.05 level (2-tailed).

3.2.3.5. MANOVA Results for Biochemical Data at Larval Stage

3.2.3.5.1. Subspecies vs. Biochemical Data

A one-way between groups multivariate analysis of variance (MANOVA) was performed to investigate subspecies differences in *Pgm* activity, glycogen and protein content data of larval stage. Three independet variables were used: glycogen content, protein content and PGM activity. The independent variable was subspecies of *A. mellifera* L. Preliminary assumption testing was conducted to check for normality, linearity, univariate and multivariate outliers, homogeneity of variance-covariance matrices, and multicollinearity, with no serious violations noted.

There is a statistically significant difference between subspecies of *A. mellifera* on the combined dependent variables of larval stage as shown in Table 84

Table 84. Multivariate Tests (d) for subspecies at larval stage.

Effect		Value	F	df	Sig.	Partial Eta Squared	Observed Power(a)
Intercept	Pillai's Trace	,910	184,811(b)	3	,000	,910	1,000
	Wilks' Lambda	,090	184,811(b)	3	,000	,910	1,000
	Hotelling's Trace	10,081	184,811(b)	3	,000	,910	1,000
	Roy's Largest Root	10,081	184,811(b)	3	,000	,910	1,000
subsp	Pillai's Trace	,229	2,414	6	,031	,115	,803
	Wilks' Lambda	,773	2,524(b)	6	,025	,121	,822
	Hotelling's Trace	,292	2,629	6	,020	,127	,840
	Roy's Largest Root	,284	5,308(c)	3	,003	,221	,914

a Computed using alpha = ,05

b Exact statistic

c The statistic is an upper bound on F that yields a lower bound on the significance level.

d Design: Intercept+subsp

Table 85. Levene's Test of Equality of Error Variances (a) for subspecies at larval stage.

	F	df1	df2	Sig.
glycogen	,942	2	57	,396
activity	,522	2	57	,596
protein	5,214	2	57	,008

Tests the null hypothesis that the error variance of the dependent variable is equal across groups.

a Design: Intercept+subsp

Table 85 indicates that variances of glycogen content and enzyme activity data are equal among subspecies (F= 0,942 and p=0.396; F=0.522, p=0.596

respectively) whereas protein content data are not equal among subspecies at larval stage (F=5,214 and p=0.008).

Table 86. Tests of Between-Subjects Effects for subspecies at larval stage.

Source	Dependent Variable	Type III Sum of Squares	df	Mean Square	F	Sig.	Partial Eta Squared	Observed Power(a)
Corrected Model	glycogen	,395(b)	2	,198	,476	,623	,016	,124
	activity	54,918(c)	2	27,459	,685	,508	,023	,160
	protein	2,812(d)	2	1,406	4,885	,011	,146	,783
Intercept	glycogen	97,767	1	97,767	235,792	,000	,805	1,000
	activity	19883,86	1	19883,86	495,959	,000	,897	1,000
	protein	72,930	1	72,930	253,418	,000	,816	1,000
subsp	glycogen	,395	2	,198	,476	,623	,016	,124
	activity	54,918	2	27,459	,685	,508	,023	,160
	protein	2,812	2	1,406	4,885	,011	,146	,783
Error	glycogen	23,634	57	,415				
	activity	2285,230	57	40,092				
	protein	16,404	57	,288				
Total	glycogen	121,796	60					
	activity	22224,01	60					
	protein	92,146	60					
Corrected Total	glycogen	24,029	59					
	activity	2340,148	59					
	protein	19,216	59					

a Computed using alpha = ,05

b R Squared = ,016 (Adjusted R Squared = -,018)

c R Squared = ,023 (Adjusted R Squared = -,011)

d R Squared = ,146 (Adjusted R Squared = ,116)

When the results for the dependent variables were considered separately, only protein content data reach statistically significant difference among subspecies (F= 4.885, p=0.011, partial eta squared= 0.146). This means that 14.6 per cent of the variance in protein content data are explained by subspecies of *A. mellifera* at larval stage as shown in Table 86.

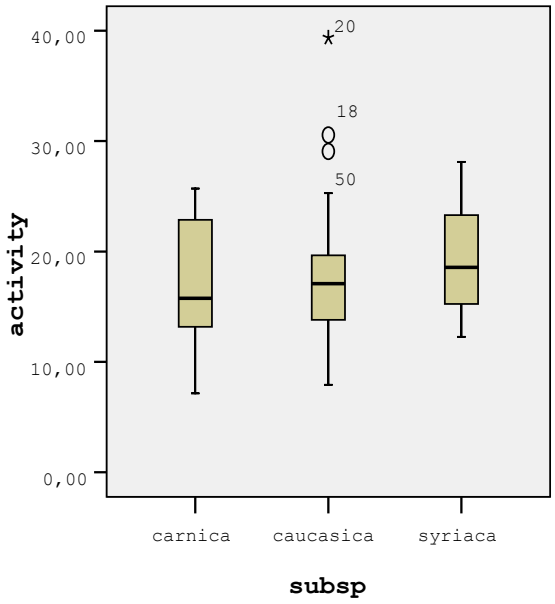


Figure 35. PGM activity versus subspecies of *A. mellifera* chart at larval stage (mean=16.94, st. dev= 6.17 for *A. m. carnica* ; mean=18.42, st.dev=7.60 for *A. m. caucasica* ; mean=19.25 st.dev=4.94 for *A. m. syriaca*).

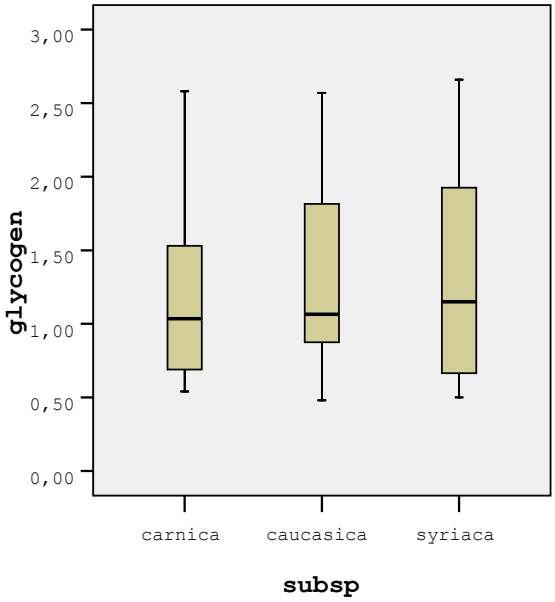


Figure 36. Glycogen content versus subspecies of *A. mellifera* chart at larval stage (mean=1.16, st. dev= 0.58 for *A. m. carnica*; mean=1.34, st.dev=0.66 for *A. m. caucasica* ; mean=1.33 st.dev=0.69 for *A. m. syriaca*).

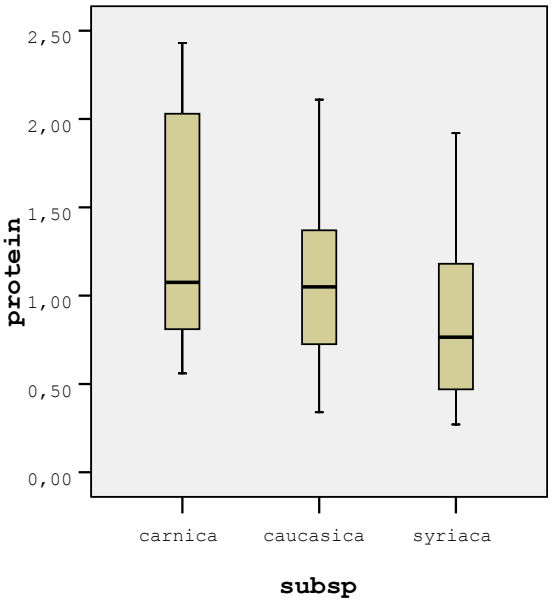


Figure 37. Protein content versus subspecies of *A. mellifera* chart at larval stage (mean=1.38, st. dev= 0.65 for *A. m. carnica* ; mean=1.09, st.dev=0.46 for *A. m. caucasica*; mean=0.85 st.dev=0.47 for *A. m. syriaca*).

3.2.3.5.2. Season vs. Biochemical Data

A one-way between groups multivariate analysis of variance (MANOVA) was performed to investigate seasonal differences in PGM activity, glycogen and protein content data of larval stage. Three independet variables were used: glycogen content, protein content and PGM activity. The independent variable was season. Preliminary assumption testing was conducted to check for normality, linearity, univariate and multivariate outliers, homogeneity of variance-covariance matrices, and multicollinearity, with no serious violations noted.

There is a statistically significant difference between seasons on the combined dependent variables of larval stage as shown in Table 87 (F=32.082, p=0.00, Wilks' Lambda=0.368, partial eta squared=0.632)

Table 87. Multivariate Tests (d) for seasons at larval stage.

Effect		Value	F	df	Sig.	Partial Eta Squared	Observed Power(a)
Intercept	Pillai's Trace	,960	446,274(b)	3	,000	,960	1,000
	Wilks' Lambda	,040	446,274(b)	3	,000	,960	1,000
	Hotelling's Trace	23,908	446,274(b)	3	,000	,960	1,000
	Roy's Largest Root	23,908	446,274(b)	3	,000	,960	1,000
season	Pillai's Trace	,632	32,082(b)	3	,000	,632	1,000
	Wilks' Lambda	,368	32,082(b)	3	,000	,632	1,000
	Hotelling's Trace	1,719	32,082(b)	3	,000	,632	1,000
	Roy's Largest Root	1,719	32,082(b)	3	,000	,632	1,000

a Computed using alpha = ,05

b Exact statistic

c Design: Intercept+season

Table 88. Levene's Test of Equality of Error Variances (a) for seasons at larval stage.

	F	df1	df2	Sig.
glycogen	2,968	1	58	,090
activity	1,255	1	58	,267
protein	17,155	1	58	,000

Tests the null hypothesis that the error variance of the dependent variable is equal across groups.

a Design: Intercept+season

Table 88 indicates that variances of protein content data are not equal among subspecies (F= 17,155 and p=0.00) whereas variances of PGM activity and glycogen content data are equal among subspecies at larval stage (F=1.255, p=0.267; F=2.968, p=0.090, respectively).

Table 89. Tests of Between-Subjects Effects for seasons at larval stage.

Source	Dependent Variable	Type III Sum of Squares	df	Mean Square	F	Sig.	Partial Eta Squared	Observed Power(a)
Corrected Model	glycogen	6,902(b)	1	6,902	23,373	,000	,287	,997
	activity	798,766(c)	1	798,766	30,056	,000	,341	1,000
	protein	8,641(d)	1	8,641	47,396	,000	,450	1,000
Intercept	glycogen	97,767	1	97,767	331,083	,000	,851	1,000
	activity	19883,865	1	19883,865	748,202	,000	,928	1,000
	protein	72,930	1	72,930	400,015	,000	,873	1,000
season	glycogen	6,902	1	6,902	23,373	,000	,287	,997
	activity	798,766	1	798,766	30,056	,000	,341	1,000
	protein	8,641	1	8,641	47,396	,000	,450	1,000
Error	glycogen	17,127	58	,295				
	activity	1541,382	58	26,576				
	protein	10,575	58	,182				
Total	glycogen	121,796	60					
	activity	22224,013	60					
	protein	92,146	60					
Corrected Total	glycogen	24,029	59					
	activity	2340,148	59					
	protein	19,216	59					

a Computed using alpha = ,05

b R Squared = ,287 (Adjusted R Squared = ,275)

c R Squared = ,341 (Adjusted R Squared = ,330)

d R Squared = ,450 (Adjusted R Squared = ,440)

When the results for the dependent variables were considered seperately, PGM activity, glycogen content and protein content data exhibit statistically significant difference between seasons at larval stage (F= 30.056, p=0.00, partial eta square=0.341; F=23.372, p=0.00, Partial eta squared=0.287; F=47.396, p=0.00, Partial eta squared=0.450, respectively). This means that 34.1 per cent of the variances in PGM activity, 28.7 per cent of the variances in glycogen content and 45.0 per cent of the variance in protein content data are explained by seasonal differentiation as shown in Table 89.

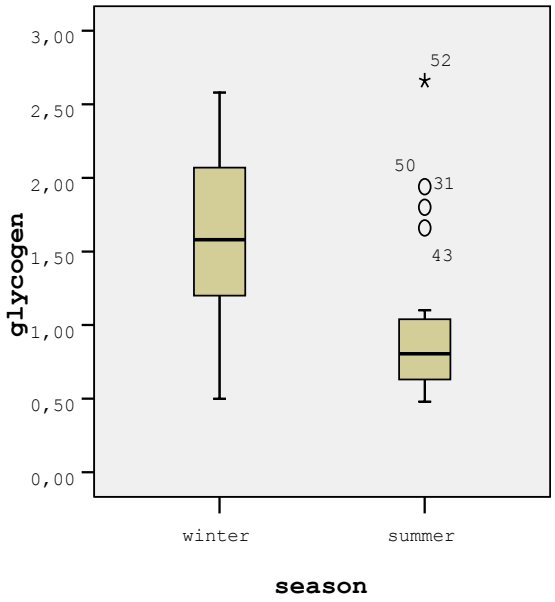


Figure 38. Glycogen content versus season chart at larval stage (mean=1.61, st. dev= 0.60 for winter season; mean=0.94 st.dev=0.49 for summer season).

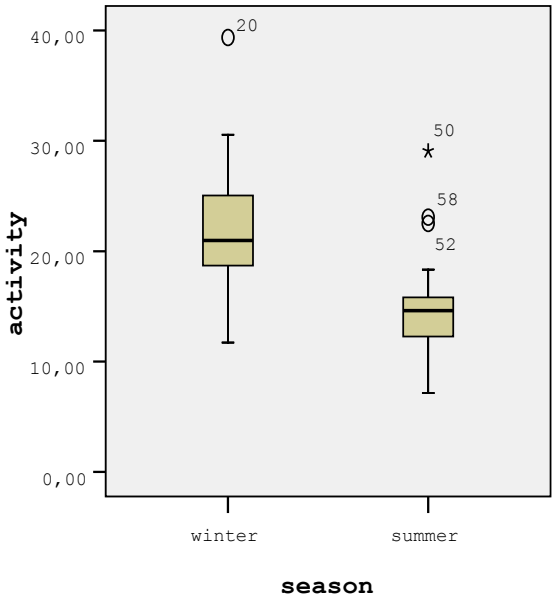


Figure 39. PGM activity versus season chart at larval stage (mean=21.85, st. dev= 5.56 for winter season; mean=14.56 st.dev=4.72 for summer season).

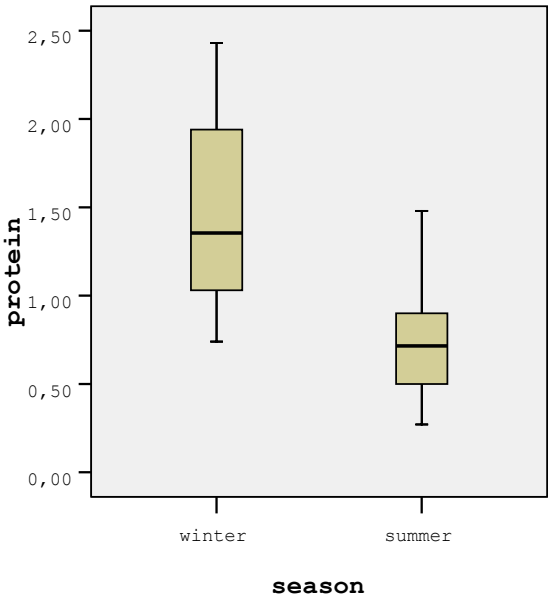


Figure 40. Protein content versus season chart at larval stage (mean=1.48, st. dev= 0.52 for winter season; mean=0.72 st.dev=0.30 for summer season).

3.2.3.5.3. Genotype vs. Biochemical Data

A one-way between groups multivariate analysis of variance (MANOVA) was performed to investigate genotypic differences in PGM activity, glycogen and protein content data of larval stage. Three independet variables were used: glycogen content, protein content and PGM activity. The independent variable was genotype. Preliminary assumption testing was conducted to check for normality, linearity, univariate and multivariate outliers, homogeneity of variance-covariance matrices, and multicollinearity, with no serious violations noted.

There is a statistically significant difference between genotypes of *Pgm* allozymes on the combined dependent variables of larval stage as shown in Table 90 (F=51.24, p=0.00, Wilks' Lambda=0.267, partial eta squared=0.733).

Table 90. Multivariate Tests (d) for genotypes at larval stage.

Effect		Value	F	df	Sig.	Partial Eta Squared	Observed Power(a)
Intercept	Pillai's Trace	,968	568,328(b)	3	,000	,968	1,000
	Wilks' Lambda	,032	568,328(b)	3	,000	,968	1,000
	Hotelling's Trace	30,446	568,328(b)	3	,000	,968	1,000
	Roy's Largest Root	30,446	568,328(b)	3	,000	,968	1,000
genotype	Pillai's Trace	,733	51,237(b)	3	,000	,733	1,000
	Wilks' Lambda	,267	51,237(b)	3	,000	,733	1,000
	Hotelling's Trace	2,745	51,237(b)	3	,000	,733	1,000
	Roy's Largest Root	2,745	51,237(b)	3	,000	,733	1,000

a Computed using alpha = ,05

b Exact statistic

c Design: Intercept+genotype

Table 91. Levene's Test of Equality of Error Variances (a) for genotypes at larval stage.

	F	df1	df2	Sig.
glycogen	25,725	1	58	,000
activity	2,842	1	58	,097
protein	,548	1	58	,462

Tests the null hypothesis that the error variance of the dependent variable is equal across groups.

a Design: Intercept+genotype

Table 91 indicates that variances of glycogen content data are not equal among genotypes (F= 25,725 and p=0.00) whereas variances of PGM activity and protein content data are equal among genotypes of larval stage (F=2.842, p=0.097; F=0.548, p=0.462 respectively).

Table 92. Tests of Between-Subjects Effects for genotypes at larval stage.

Source	Dependent Variable	Type III Sum of Squares	df	Mean Square	F	Sig.	Partial Eta Squared	Observed Power(a)
Corrected Model	glycogen	15,616(b)	1	15,616	107,660	,000	,650	1,000
	activity	1482,054(c)	1	1482,054	100,175	,000	,633	1,000
	protein	2,372(d)	1	2,372	8,168	,006	,123	,802
Intercept	glycogen	97,767	1	97,767	674,019	,000	,921	1,000
	activity	19883,865	1	19883,86	1343,984	,000	,959	1,000
	protein	72,930	1	72,930	251,131	,000	,812	1,000
genotype	glycogen	15,616	1	15,616	107,660	,000	,650	1,000
	activity	1482,054	1	1482,054	100,175	,000	,633	1,000
	protein	2,372	1	2,372	8,168	,006	,123	,802
Error	glycogen	8,413	58	,145				
	activity	858,094	58	14,795				
	protein	16,844	58	,290				
Total	glycogen	121,796	60					
	activity	22224,013	60					
	protein	92,146	60					
Corrected Total	glycogen	24,029	59					
	activity	2340,148	59					
	protein	19,216	59					

a Computed using alpha = ,05

b R Squared = ,650 (Adjusted R Squared = ,644)

c R Squared = ,633 (Adjusted R Squared = ,627)

d R Squared = ,123 (Adjusted R Squared = ,108)

When the results for the dependent variables were considered seperately; glycogen content, PGM activity and protein content data exhibit statistically significant difference among genotypes at larval stage (F= 107.66, p=0.00, partial eta square=0.65; F=100.18, p=0.00, partial eta square= 0.633; F=8,168, p=0.006, partial eta square=0.123). This means that 65.0 per cent of the variance in glycogen content data and 63.3 per cent of the variance in enzyme activity data and 12.3 per cent of the variance in protein content data are explained by seasonal differentiation in larval stage as shown in Table 92.

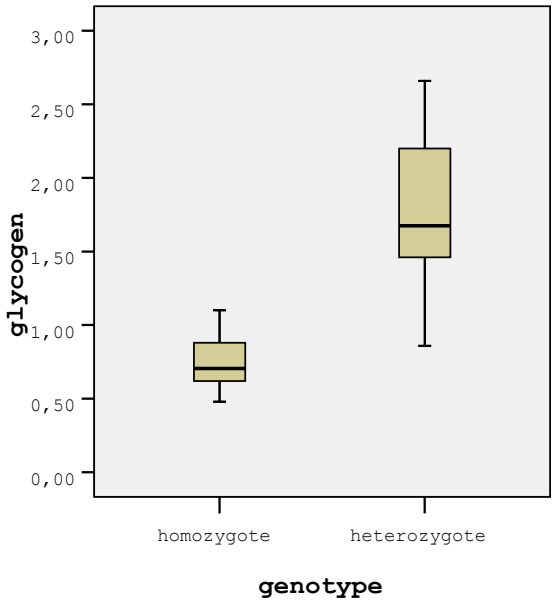


Figure 41. Glycogen content versus genotype chart at larval stage (mean=0.77, st. dev= 0.19 for homozygotes; mean=1.79 st.dev=0.50 for heterozygotes).

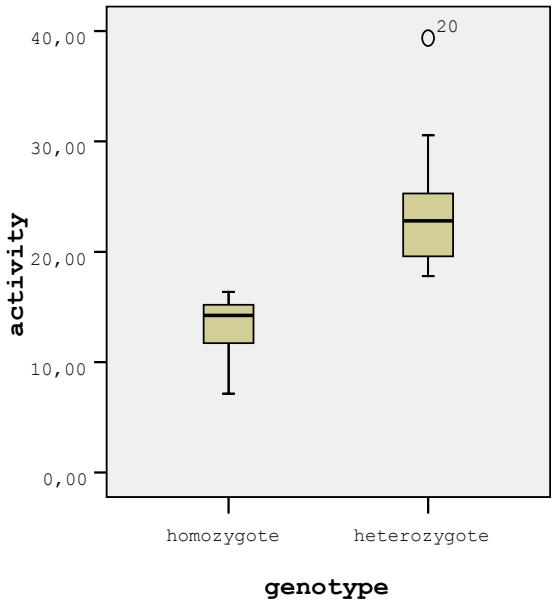


Figure 42. PGM activity versus genotype chart at larval stage (mean=13.23, st. dev= 2.87 for homozygotes; mean=23.17 st.dev=4.62 for heterozygotes).

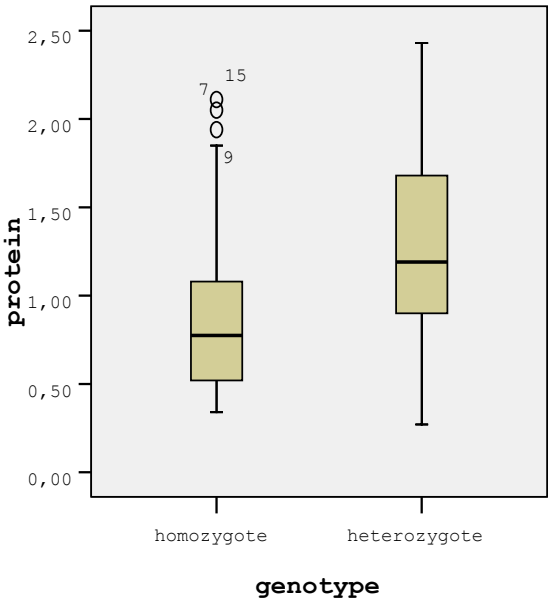


Figure 43. Protein content versus genotype chart at larval stage (mean=0.90, st. dev= 0.51 for homozygotes; mean=1.30 st.dev=0.57 for heterozygotes).

CHAPTER 4

DISCUSSION

In evolutionary genetics, scientist always wonder if naturally emerged allozyme or amino acid polimorphisms are accompanied by significant physiological variation (Watt 1994; Mitton 1998; Eanes 1999). This is the first study that demonstrates the correlation between seasonal variation at Pgm locus and biochemical variables at developmental stages in three subspecies of honeybee, *A. m. carnica, A. m. caucasica* and *A. m. syriaca*. As enzyme polymorphism may account for the adaptation of natural populations under different environmental conditions (Verrelli and Eanes, 2000), three subspecies of honeybee from ecologically different locations of Turkey (Kırklareli, Hatay, Artvin) was investigated in electrophoretic and biochemical studies. A long time analysis (18 months) was performed to identify and characterize enzyme polymorphism at *Pgm, Pgi, Hk* and *G6pd* loci which undertake key roles in energy metabolism of honey bees.

To determine and relate enzyme polymorphism and biochemical correlates; genotypic, seasonal and ontogenetic differentiation of enzyme activity, protein and glycogen content of these three subspecies were investigated. And it is found that heterozygote (*Pgm-75/100*) and homozygote (*Pgm-75/75*) individuals have different characteristics at each developmental stage studied. In addition to this, a strong positive correlation was detected between PGM activity and glycogen content at each developmental stage. Thus, results of these study reveal that *Pgm* polymorphism has adaptive value in terms of different climatic and ecological conditions at biochemical level.

4.1. Seasonal Variation at Allozyme Level

Only *Pgm* showed a seasonal variation at allozyme level among four enzymes studied. Frequencies of common alleles (Pgm-75 and *Pgm*-100) of *Pgm* exhibited seasonal variation in a range of 0.5-1.0. and 0.0-0.5, respectively. The genotype frequencies of spring and summer samples were found to be in Hardy-Weinberg equilibrium whereas the genotype frequencies of winter samples exhibited significant levels of deviations in each subspecies studied. Also, the obvious differences 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 winter months. This result indicates violation assumption(s) of Hardy-Weinberg equilibrium and this situation may be a sign of inbreeding. Because queen do not lay eggs during winter until the beginning of spring.

One important finding of this study is that fluctuation of genotype frequencies of *Pgm* heterozygotes and homozygotes found to be in accordance with the temperature fluctuations of the local climate. When temperature fluctuation shifts in one location to another, the genotype frequencies of heterozygotes and homozygotes shift in line with temperature. Because northwestern, northeastern and southeastern Anatolia exhibit different climatic and ecological conditions in terms of average temperature and precipitation, the subspecies adapted to these locations have different timing in terms of monthly genetic variation at allozyme level.

From the ontogenetic perspective, each developmental stage exhibited the same pattern of seasonal genotype fluctuations at *Pgm* locus. However allozyme variants of *Pgm* at egg stage could not be detected on starch gel, this continuing pattern throughout the ontogenesis reveals that adaptation to climate occur not only at adult stage but in earlier stages of development. As the same pattern was observed except egg stage, the possibility should be investigated

that selection against homozygotes may occur with the arrival of winter at egg stage and then eggs having homozygous genotypes at *Pgm* locus are eliminated with a behavioral adaptation before winter.

In *A. m. carnica* population, which is adapted to temperate climate with dry summers and moderate precipitation in the rest of the year, genotype frequency of heterozygotes dropped down below 0.05 level in August. First sharp change occurs in October at the middle of autumn when temperature starts to decrease and a very slight increase continues and eventually reaches its maximum with 0.975 level in March. Then the second sharp change was seen in April and the genotype frequencies of heterozygotes decreased steeply at the beginning of spring when temperatures starts to increase.

In *A. m. caucasica* population, which is adapted to subarctic climate with harsh winters and a long snow cover up to eight months, genotype frequency of heterozygotes drops down below 0.05 level in August. First sharp change occurs in September at the beginning of autumn when temperature starts to decrease and a slight increase continues and reaches its maximum with 1.00 level in February. Then the second sharp change is seen in May and the genotype frequencies of heterozygotes decreases steeply at late spring when temperature eventually starts to increase.

In *A. m. syriaca* population, which is adapted to subtropical Mediterranean climate with warm temperatures throughout the year and high precipitation rates in winter, genotype frequency of heterozygotes drops down below 0.1 level in August. After it exhibits slight increase however a sharp increase is seen in November and continues to increase slightly and eventually reach its maximum with 0.975 level in February. Then a sharp decrease is seen in April and the genotype frequency of heterozygotes continue to decrease slightly until August. Because in Hatay as the temperature drops down sharply at late autumn, the genotype frequency of heterozygotes increase and homozygotes

decrease sharply in November. And the second sharp change in genotype frequencies occur in April when the temperature is much higher in contrast with March temperatures.

In contrast to *Pgm* locus, the frequencies of *Hk* genotypes and alleles did not differed seasonally in all locations throughout the year. Also, the genotype frequencies of all samples were found to be in Hardy-Weinberg equilibrium. The heterozygosity values remained stable throughout the year at *Hk* locus and a significant deviation from expected heterozygosities was not observed indicating that observed heterozygosity values are in accordance with the ones estimated under random mating conditions.

G6pd and *Pgi* loci found to be monomorphic, exhibits no electrophoretic variants throughout the year in electrophoretic analysis in each subspecies studied. The reason of monomorphism in these loci may be explained by conservation at these loci due to their important and common metabolic role in many organisms. Also, it should be taken into account that each variation at amino acid level may not be seen as allozyme variants.

Some enzyme loci respond to seasonal fluctuations of ecological parameters. In this study, no such pattern was observed except *Pgm* locus. Eanes (1999) published data supporting the hypothesis that electrophoretic variation is generally associated with geographic variation due to changing ecological characteristics and he observed a wide geographical variation in allozyme polymorphism for glycolytic enzymes in *D. melanogaster*. Therefore, it should be taken into account that without the knowledge of climatic and ecological characteristics of the sampling area and sample collection time, erroneous inferences can be made about population structure by assuming that allozyme frequencies are stable throughout seasons in an enzyme locus.

Temporal variations in allele and genotype frequencies at an enzyme locus may result from inadequate sampling such as low sample size or non-random sampling (Cavener and Clegg, 1981). However, in this study a total number of 2160 honeybees were used in electrophoretic analysis and honeybees were sampled regularly for 18 months from all colonies to avoid consequences of low sample size. Also, samples were collected in the same fashion by the same person during sampling process. Thus, results of this study which demonstrates seasonal variation in allele and genotype frequencies at *Pgm* locus are reliable scientifically.

A variety of hypothesis proposed to account for the underlying mechanisms of enzyme polymorphism and seasonal variation at a locus. These mechanisms can be summarized as nucleotide substitutions at structural gene locus, differential gene expression, genetic linkage, transcriptional (alternate transcription) or post-translational (alternative splicing, alternate translation, co-translational and post-translational) modifications (Finnerty and Johnson, 1979; Poly, 1997). Post-translational modifications may alter the electrophoretic mobility of the modified enzyme by affecting a fraction of enzyme or may cause additional staining band to appear on gels which in turn leads to misinterpretation of the gel (Poly, 1997). Also, the same author reported that ontogenetic and tissue-specific expression may result from post-translational modifications instead of gene control or restricted expression, respectively.

According to Neangarder et al. (2003) there is a linkage between *Pgi* and heat shock proteins. *Pgi* genotypes of mountain leaf betle, *Chrysomella aeneicollis*, have exhibited differential heat-shock protein expression profiles which in turn caused functional and physiological differences among *Pgi* genotypes and these functional differentiation affected the fitness of individuals via changing their response to extreme temperatures (Dahlhoff and Rank, 2000; Neangarder

et al. 2003). There can be a similar phenomenon at *Pgm* which should be investigated.

As far as *Pgm* concerned, all of these possibilities that may lead to enzyme polymorphism and seasonal variation at *Pgm* locus should be investigated to make more accurate correlations between enzyme polymorphisms and physiological variables. For example, seasonal variation in *Pgm* allele frequencies in honeybees may result from the differential expression of regulatory locus which controls the expression of the different *Pgm* alleles in response to the changes in temperature. As temperature fluctuates between winter and summer conditions, regulatory locus may be activated or inactivated and in turn causes of this cycling *Pgm* allele frequencies in accordance with the seasonal changes.

4.2. Ontogenetic Survey of Biochemical Correlates

As physical activity, division of labor, food availability differ among developmental stages and age, a statistically significant difference was observed among developmental stages of honey bees in terms of *Pgm* activity, glycogen and protein content of samples (F=139.36, p=0.000, Wilky's Lambda=0.087) by performing a one-way between groups multivariate analysis of variance (MANOVA). After acquiring this knowledge of stage-specific differentiation of biochemical variables, intra-stage investigations were conducted concerning relationships among biochemical variables.

4.2.1 Biochemical Correlates at Adult Stage

As far as adult stage is concerned, MANOVA analysis revealed that there is approximately two-fold increase in PGM activity in *Pgm*-75/100 genotype when compared to *Pgm*-75/75 genotype . This result indicates that enzyme variants are different. Accordingly, there is approximately two-fold increase in

glycogen content in *Pgm*-75/100 . Protein content did not differ between genotypes (p=0.658, F=0.198, Partial eta squared=0.003).

When seasonal effects were analysed, it was found that PGM activity (mean=111.9 for winter, mean=69.09 for summer), glycogen content (mean=2.48 for winter, mean=1.54 for summer) and protein content (mean=4.15 for winter, mean=2.72 for summer) exhibited seasonal differentiation with approximately1.6 fold increase in winter season.

Among subspecies only PGM activity differentiated (mean=110.52 *A. m. carnica*, mean=78.26 for *A. m. caucasica* , mean=85.65 for *A. m. syri*aca). Mean values indicate that in *A. m. caucasica* and *A. m. syriaca* populations have similar PGM activity levels whereas *A. m. carnica* population has significantly higher PGM activity. This difference may result from the ecological conditions or more possibly as not equal number of heterozygotes and homozgotes used in the analysis. According to the assay procedure first biochemical data acquired from samples to avoid any decrease in enzyme activity and then genotypes of samples were detected.

Furthermore, it was observed that PGM activity is an important determinant of glycogen content of the bees at adult stage since there is a positive strong correlation between enzyme activity and glycogen content in all populations representing *A. m. carnica, A. m. caucasica,* and *A. m. syriaca* (Pearson r=0.723, p=0.00; r=0.817, p=0.00 and r=0.838, p=0.00, respectively). Protein content is independent of PGM activity and glycogen content since there is no correlation between PGM activity and protein content, and glycogen content and protein content. Therefore, it can be concluded that enzyme activity and glycogen content of the bees follow a similar seasonal pattern to the shifts in genotype frequencies at *Pgm* locus in each of the subspecies at adult stage. This result indicates that as an important branch point enzyme between glycolytic pathway and the pentose shunt, genetic differences at *Pgm* locus

lead to functional consequences in terms of enzyme activity which in turn can influence energy metabolism and glycogen content of honeybees. Thus, fitness of individual bees is highly influenced by their genotype at *Pgm* locus due to seasonal conditions.

4.2.2 Biochemical Correlates at Pupal Stage

As far as pupal stage is concerned, MANOVA analysis revealed that there is 1.7-fold increase in PGM activity in *Pgm*-75/100 genotype when compared to *Pgm*-75/75 genotype. This result indicates that enzyme variants are different. Accordingly, there is approximately 1.4-fold increase in glycogen content in *Pgm*-75/100. Also, there is 1.2 fold increase in protein content in *Pgm*-75/100 genotype.

When seasonal effects were analysed, it was found that only PGM activity and glycogen content (mean=2.04 for winter, mean=1.29 for summer). exhibited significant seasonal differentiation with approximately 1.2 and 1.6 fold increase in winter season, respectively.

PGM activity were different among subspecies. This difference may result from the ecological conditions or possibly emerged from unequal number of heterozygotes and homozgotes used in the analysis. According to the assay procedure first biochemical data was acquired from samples to avoid any decrease in enzyme activity and then genotypes of samples were detected.

Furthermore, it was observed that PGM activity is an important determinant of glycogen content of the bees at pupal stage since there is a positive strong correlation between enzyme activity and glycogen content in *A m. carnica*, *A. m. caucasica* and *A. m. syriaca* populations (Pearson r=0.733, p=0.00; r=0.714, p=0.00 and r=0.821, p=0.00, respectively). Also, it was found that there is a negative correlation between protein content and PGM activity and protein and

glycogen content in *A. m. carnica* and *A. m. syriaca* populations. This result can be interpreted that these variables are independent. Therefore, it can be concluded that enzyme activity and glycogen content of the bees follow a similar seasonal pattern to the shifts in genotype frequencies at *Pgm* locus in each of the subspecies at pupal stage. This result indicates that as an important branch point enzyme between glycolytic pathway and the pentose shunt, genetic differences at *Pgm* locus lead to biochemical consequences in terms of enzyme activity which in turn can influence glycogen content, energy metabolism and fitness of honey bee pupae.

4.2.3 Biochemical Correlates at Larval Stage

As far as larval stage is concerned, MANOVA analysis revealed that there is 1.7-fold increase in PGM activity in *Pgm*-75/100 genotype when compared to Pgm-75/75 genotype which indicates that enzyme variants are biochemically different. Accordingly, there is approximately 2.3-fold increase in glycogen content and 1.4 fold increase in protein content in Pgm-75/100 genotype.

When seasonal effects were analysed, it was found that PGM activity, glycogen content, and protein content exhibited significant seasonal differentiation with approximately 1.5, 1.7 and 2-fold increase in winter season, respectively. But among subspecies only protein content exhibited differentiation. Furthermore, it was observed that PGM activity is an important determinant of glycogen content of the bees since there is a strong correlation between enzyme activity and glycogen content in all populations studied (Pearson r=0.781, p=0.00; r=0.823, p=0.00 and r=0.809, p=0.00, respectively). Also, positive correlation was found between protein content and PGM activity in *A. m. carnica* and *A. m. syriaca* populations. Since no information was reported in literature concerning a relationship between total soluble protein content and energy metabolism, these results can not be interpreted as protein content is a determinant of glycogen content or PGM activity.

It can be concluded that enzyme activity and glycogen content of the bees follow a similar seasonal pattern with the shifts in genotype frequencies at *Pgm* locus in each of the subspecies larval stage. This result indicates that as an important branch point enzyme between glycolytic pathway and the pentose shunt, genetic differences at *Pgm* locus lead to changes of enzyme activity which in turn can influence glycogen content, energy metabolism, and fitness of honey bee larvae.

The relationship among biochemical data may be interpreted by looking from a greater perspective. As MANOVA results revealed, PGM activity changes among developmental stages. There is a trend of increase from larval stage to adult stage. Accordingly, glycogen content levels follow the same trend among developmental stages. Whereas, pupae have the highest, larvae have the lowest protein content.

Observation of different enzyme activity levels among developmental stages may be due to differential gene expression, or transcriptional/post-transcriptional modifications that may be the underlying mechanisms of enzyme polymorphism. Natural selection may be acting on this polymorphism related to the metabolic process.

Yamate and Yamazaki (1999) had observed allozymic polymorphism and differential specific activity due to food availability and developmental stages of *D. melanogester* at *α-Amylase* locus and found that food-dependent activity change is regulated at transcriptional level and stage-dependent activity is largely explained by differences in mRNA levels. A similar mechanism may be causing differential PGM activity levels in honey bees.

The glycogen molecule functions as the secondary long-term energy storage in animal cells and can be quickly mobilized to meet a sudden need for glucose.

PGM plays a crucial role in glycogen metabolism by functioning at a branch point of the glycolytic pathway dividing into the glycogen synthesis, the pentose shunt, and the main glycolytic corridor and catalyzes the reversible transfer of a phosphate group between glucose-1-phosphate and glucose-6-phosphate, the first intermediate in glycolysis (Lehninger, 2000). Verelli and Eanes (2001) reported that changing concentrations of glucose-6-phosphate may cause inhibition or activation of other enzymes in the glycolysis due to competition at the branch points and controls the increase or decrease flux through glycogenesis. Although the complete mechanism is not known the results of this and other studies (Watt 1983; Dahlhoff and Rank, 2000) concerning genetic variation at *Pgm* locus indicate that enzyme polymorphism may affect metabolic pathways.

Kacser and Burns (1981) pointed out that the enzymes are part of the system, a flux, and they are interacting that the electrophoretic variants differ considerably they can not lead large changes in metabolic flux and a heterozygous would have only half of the activity for a given enzyme. The results of this study do not support this hypothesis. The correlation between Pgm allozyme polymorphisms, PGM activity and glycogen content can be interpreted as high PGM activity is accompanied by higher flux to glycogen synthesis. So, these results may suggest that allozyme polymorphism is under selection at pentose shunt and may be favored for increased metabolic storage together with adaptation to different environmental conditions.

This study indicates that frequency of heterozygotes increase in winter and this increase is accompanied by higher PGM activity and higher glycogen content at different developmental stages. This finding may be related to longer life span of winter bees. It can be said that during winter flight and foraging tasks are not performed and so winter bees do not consume their glycogen storage. But this hypothesis can not be supported because when summer bees perform tasks such as by foraging, winter bees perform the task of thermoregulation by

forming clusters which has a similar metabolic cost to foraging flights of summer bees (Panzenböck and Crailsheim, 1997). On the other hand, increased heterozygosity at *Pgm* locus may support the efficient use of glycogen storage in order to maintain thermoregulation and longevity of winter bees which is crucial for survival of colony in winter when brood production decreases.

CHAPTER 5

CONCLUSION

This is the first study that demonstrates correlation between seasonal variation at *Pgm* locus and biochemical variables at different developmental stages in three subspecies of honeybee (*A. mellifera* L.), which are adapted to different climatic and ecological conditions. Analysis of enzyme activity and glycogen content revealed that there are differences at genotypes, seasons and developmental stages. The heterozygote (*Pgm-75/100*) and homozygote (*Pgm-75/75*) individuals have different characteristics at each developmental stage studied meaning that *Pgm* polymorphism has adaptive value in terms of different climatic and ecological conditions at biochemical and possibly at functional level which needs to be investigated.

In further analysis, gene expression profiles of winter and summer bees should be analyzed in order to understand the molecular genetic mechanisms of seasonal shifts in *Pgm* genotype frequencies. From a greater perspective, other enzymes at the branch point of glycolytic pathway can be investigated in terms of enzyme activity and amino acid polymorphism so that energy metabolism of *A. mellifera* L. can be elucidated.

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APPENDIX A

REAGENTS

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

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

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 (450ml)	BioRad -500-0006
5	Bovine Serum Albumin Standard	Sigma-A2153
6		

APPENDIX B

EQUIPMENT

	Name of the Reagent	Brand Name / Model
1	Multipor II Electrophoresis Unit	Amersham
2	Power Supply	Amersham/ EPS 3501
3	Cooling Device	Heto
4	UV/Visible Spectrophotometer	Cary 100
5	Microplate Reader	BioRad/ 680
6	Centrifuge	Eppendorf/5415R
7	pH meter	Eutech/Cyberscan 500

APPENDIX C

SYMBOLS

PGM : Phosphoglucomutase
HK : Hexokinase
PGI : Phosphoglucose isomerase
G-6-PDH: Glucose-6-Phosphate Dehydrogenase
G-1-P : Glucose-1-Phosphate
G-6-P : Glucose-6-Phosphate
$MgCl_2$: Magnesium chloride
NADP : Nicotine amide adenine dinucleotide phosphate
Mg : Magnesium
PMS : Phenasine metosulphate
Tris : Tris [Hydroxymethyl]-aminomethane
HCl : Hydrochloric acid
ATP : Adenosine triphosphate
MTT : 3-(4,5-Dimethyl-2-Thiazyl)-2,5-Diphenyl-2H-Tetrazolium Bromide