SPECIFIC ACTIVITY OF PHOSPHOFRUCTOKINASE IN FLIGHT MUSCLE OF
HONEY BEES (Apis mellifera L.) IN WINTER, SUMMER,
AND IN A FLIGHT ROOM

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Abstract

1. The maximal activity of phosphofructokinase (PFK) has been measured in flight muscle of honey bees (*Apis mellifera* L.) in summer and winter. A flight room was also used to test PFK activity in flight muscle of winter bees kept on summer photoperiod and temperature. The activity of this enzyme was used to compare estimates of maximal rates of aerobic respiration in flight muscle under the conditions of the experiment.

2. PFK activity expressed per unit muscle tissue is not significantly different between summer, winter, and flight room bees. However, when reduced to per unit muscle protein, flight room bees have significantly less PFK activity than summer and winter bees.

3. There is no relationship between PFK and aging in bees since the specific activity of PFK remains the same in winter and summer seasons. The fact that the specific activity in flight room bees is lower than other bees tested indicates that these bees are different than summer or winter bees.
Thesis Title: Specific activity of phosphofructokinase in flight muscle of honey bees (Apis mellifera L.) in winter, summer and a flight room.

1. Key Citations


2. Keywords.

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Specific Activity of Phosphofructokinase in Flight Muscle

of Honey Bees (*Apis mellifera* L.) in Winter, Summer

and in a Flight Room
**Introduction**

Of the 20,000 species of bees in the world (Hymenoptera: Apoidea), only the four species of honey bees (sub family Apinae) and 300 species of stingless bees (sub family Meliponinae) live in permanent perennial colonies (Johansson and Johannson, 1979; Roubik, 1987). The majority of the 4,500 bee species occupying temperate regions are solitary. Of these species, the European honey bee (*Apis mellifera*), is the only social insect which does not diapause in winter, and is adapted to a much wider range of climatic conditions than other species of insects because of its permanent colonial organization (Free and Spencer-Booth, 1958; Gates, 1914; Johannson and Johannson, 1979; Ruttner, 1988).

**Summer and winter honey bees**

Honey bee populations in temperate climates follow a perennial cycle of life history. This includes seasonal changes in lifespans, physiological function, and duties performed by worker bees i.e., (division of labor). The seasonal changes are more pronounced as the distance from the equator increases (Kefuss, 1978). When spring season begins in the temperate climates, lifespans of worker bees begin to decline (Fukuda & Sekiguchi, 1966). Lifespans are
shortest, 30 to 50 days, in late spring and early summer (Free and Spencer-Booth, 1959; Sakagami & Fukuda, 1968). Following the summer season, which involves active foraging, lifespans and physiology change in worker bees giving rise to winter bees (Maurizio, 1946). Lifespans for winter bees in Sapporo, Japan average 154 days with a range of 150 to 250 days (Sakagami and Fukuda, 1968). Lifespans as long as 350 days have been recorded (Maurizio, 1959 in Rockstein, 1964). Physiological changes associated with the difference between winter and summer bees can be seen in the fat body which stores lipids, proteins, and carbohydrates, and in the hypopharyngeal glands used in larval feeding. Fully developed hypopharyngeal glands and fat bodies are associated with increased lifespans (Haydak, 1957; Ribbands, 1953) and thus would be expected in winter bees. Changes in the hypopharyngeal gland and fat body may be attributed to the nutritional status of the colony which in turn determines the amount of worker bees employed in various duties (Shehata, et al, 1981). Ribbands (1952) reported that the food supply may control the hypertrophy in hypopharyngeal glands. He found that the hypopharyngeal glands of nurse bees enlarged when they were given suitable protein. Prolongation of hypopharyngeal gland function has been shown with decreased or absence of brood rearing (Butler, 1984; Haydak, 1957; Maurizio, 1950; Neukirch, 1982, Rockstein, 1964). The degeneration of the hypopharyngeal
glands may be directly under juvenile hormone control. This hormone is responsible for the physiological changes associated with polyethism (age related tasks) in honeybees. Increased titres of this hormone cause house bees to become field bees with a resultant decrease in longevity (Rutz, et al, 1976).

Seasonal factors may affect the course of senescence and account for winter bees living three to eight times longer than summer bees. For example, seasonal and/or genetic factors affect lifespan and determine the time of onset, course, and duration of the process of senescence (Clark, 1964; Rockstein, 1964; Sohal 1976). Also, environmental conditions and hazards specific to a season may quantitatively reduce the probability of survival.

Environment

Classical studies done by Allen (1958) and Johansson and Johansson (1979) stated that environmental factors such as temperature can affect the lifespan of the worker bee because, like other insects, it is ectothermic and its metabolic rate is temperature dependent. Further, the aging of insects may be faster at higher temperatures because of a more rapid accumulation of harmful metabolic by-products and/or loss of irreplaceable materials. Smith (1953), found that lower temperatures reduced the metabolic rate of insects. Edwards (1953) reported that metabolic rate is
inversely related to the duration of life in some species of insects. For example, Loeb and Northrop (1917) experimenting with *D. melanogaster* found that these flies maintained from emergence at a high temperature lived a shorter time than those maintained at a lower temperature.

An isolated individual worker honey bee is immobilized by low temperature at 9°C to 10°C, and killed in a few hours at about -3°C (Free and Spencer-Booth, 1960). A group of worker bees is able to control environmental conditions in summer and winter only as a result of their cooperative social behavior (Corkins, 1932; Gates, 1914; Ribbands, 1953; Southwick, 1982; Southwick and Mugaas, 1971). In temperate climates during winter, most species of insects diapause (Salt, 1969), but honey bees form a dense cluster of 16,000 to 40,000 worker bees and one queen in a natural or manmade hive (Corkins and Gilbert, 1932; McLellan, 1978; Nolan, 1925; Phillips, et al, 1914; Southwick, 1983). This cluster maintains a "summer heat" during the coldest months of winter (Bevan, 1827), and becomes more compact with decreasing cold temperature thereby reducing its surface area and heat loss. The cluster can maintain central core temperatures of 18°C to 35°C over long term exposure to very low air temperatures (Corkins, 1930; Free and Simpson, 1963; Gates, 1914; Ritter, 1982, Southwick, 1982). The source of this heat is individual workers in the cluster which shiver
their flight muscles in their thoraxes (Corkins and Gilbert, 1932; Esch, 1960, 1988, 1990; Esch and Bastian, 1964; Free and Spencer-Booth, 1959; Southwick and Mugaas, 1971).

Carbohydrates are the source of metabolic energy for thermogenesis in winter bees (Southwick, 1985; Storey, 1985). Shivering increases metabolic rate and converts chemical energy into heat because of biochemical inefficiencies (Josephson, 1981). When brood is also present, the cluster maintains the core temperature at 33°C to 36°C which is necessary for incubation (Fahrenholz et al., 1989; Gates, 1914; Wilson & Milum, 1927; Free & Simpson, 1963). Studies of the general response of energy metabolism of honey bee clusters to a range of environmental temperatures have shown that metabolic energy increases with falling temperatures below about 5°C, and with rising temperatures above 10°C (Gilbert and Corkins, 1932; Ritter, 1982, Southwick, 1982, 1983, 1988).

Control of nest conditions by worker bees during warm summer months occurs when inside nest temperatures exceed 35°C. Worker bees are able to cool the colony by fanning their wings (Heinrich, 1980; Lindauer, 1954). Bees will expend great amounts of energy to keep the nest at the proper temperature, because an increase of 1°C to 2°C above 36°C can harm larvae (Seeley and Heinrich, 1981). If fanning is not enough to cool the colony, foragers, in addition to fanning, bring water to the nest and place droplets on the
Humidity and its effects on worker bee life span was studied by Woodrow (1935), who reported that worker bees maintained at 25% to 73% relative humidity showed no difference in life span. However, at 93% relative humidity, the life span was reduced by 75% and was most likely the result of nosema disease. Studies of the effects of high temperatures and relative humidity by Free and Spencer-Booth (1962) have shown that at high temperatures (40°C to 50°C) bees survive only short periods with high relative humidity. The survival duration was increased when the relative humidity was decreased with the same high temperatures. The reason for this is that the bees are better able to evaporate water at lower relative humidity. At lower temperatures, bees can survive a high relative humidity because desiccation becomes the major factor. Honey bee clusters are able to control humidity within the hive by increasing or decreasing the temperature and ventilation within the hive (Johansson and Johansson, 1979).

Brood rearing

Brood rearing affects longevity of worker bees. The short lives of workers emerging in mid-summer may be associated with less demand for brood rearing occurring during June or July. This is when honey bee colonies reach
maximum size and larvae:worker ratios are lowest (Free, 1966; Jeffree, 1955). The decreased demand for brood rearing encourages worker bees to leave the hive and forage for food at a younger age. Foraging bees use their flight muscles extensively, greatly increasing their metabolic rates, however, the amount of work that a forager does is of little importance in longevity aside from foraging being hazardous. Ribbands (1952), found that worker bees lived longer when they began foraging later in life. Neukirch (1982), suggested that a difference between winter and summer worker bees' lifespan may be dependent on the amount of time a worker remains in the hive (hive period) before foraging. During this relatively quiet period, workers feed larvae, maintain cells, produce wax, and distribute food (Gary, 1984). Haydak (1957) reported that worker bee hypopharyngeal glands develop quickly in summer bees to fullness by the fourth day of adult life. These glands secrete a nutritious substance (royal jelly) which is then fed to larvae and the queen bee. During the summer season, fully developed hypopharyngeal glands remain active until about the ninth day when degeneration of the glands begins. Fully developed hypopharyngeal glands are associated with increased lifespans in honey bees (Ribbands, 1953) and many overwintering worker bees have been found with fully developed functional hypopharyngeal glands at 69 days of age (Maurizo, 1954).
Experiments with worker bees caged with no queen, which relieves them from brood rearing responsibilities, have shown fully developed functional hypopharyngeal glands up to 57 days of age (Rockstein, 1964). Some of these longer lived experimental bees had fully developed glands as long as 185 days of age. It was also noted that queenless caged worker bees lived two to three weeks longer than worker bees in queenright colonies which would expose them to brood rearing responsibilities.

Maurizio (1950) found that brood rearing by worker bees causes the protein stored within the hypopharyngeal glands and fat body to become rapidly depleted. When this happens, the lifespan of the bee becomes shortened.

Diet

Diet can modify the life span in worker bees. Life spans of summer worker bees can be increased to at least 166 days by feeding them pollen early in adult life and also freeing them from brood rearing duties (Rockstein, 1964). It is unknown whether these bees were involved in flight activities or not. These experimental summer bees developed fully developed hypopharyngeal glands and a winter fat body. Reduced demands on food reserves results in prolonged maintenance of the fat body and increased life span. Fat body development in worker bees occupying northern habitats undergo large seasonal changes which may be a result of the
nutritional status of the colony (Ribbands, 1952; Shehata, et al, 1981). Ribbands (1953), states worker bees in Autumn begin to eat more pollen than is needed for brood rearing which resulted in a greater development of the fat bodies and hypopharyngeal glands which may help to prolong their lives.

Hormones

Perhaps the hormone which is most widely known and studied in honey bees is juvenile hormone which is found to be a very important factor in the aging process. Increasing levels of this hormone occur in the transformation of young into old honey bees (Rutz, et al, 1976). Experimentation by Rutz, et al, (1974, 1976) found that by injecting high doses of juvenile hormone into young hive bees changed their physiology into that of older field bees, thus decreasing their lifespan. Therefore, it is reasonable that this hormone and/or other hormones are important in senescence.

Flight metabolism

Honey bee flight is aerobic and extremely energy demanding (Beenakkers, et al, 1984; Nachtigal, 1989; Sactor, 1965; Storey, 1985). Flight normally occurs during warm summer months when worker bees forage for nectar, pollen, water and propolis (tree sap). A comparison of metabolic rate during flight with the maximal activities of key
regulatory enzymes shows that flight requires the complete use of the enzymatic potential in the flight muscles (Crabtree and Newsholme, 1975). The rate of substrate oxidation in insect flight muscle may increase by more than 100 times the resting rate when an insect begins flight (Sactor, 1975). The corresponding 20 to 100 fold increase in oxygen consumption for insect flight compares to a seven to ten fold increase for aerobic work performed in mammalian muscles (Storey, 1985). The temperature of an isolated resting honey bee is close to environmental temperature, but the same bee when actively flying, produces temperatures in excess of 20°C above ambient temperatures (Free and Spencer-Booth, 1962; Kammer and Heinrich, 1974; Heinrich, 1979).

Honey bees are believed to use only carbohydrates during flight which occurs from spring to late fall (Beenakkers, 1969; Nachtigal, 1989; Sactor, 1965; Storey, 1985). Carbohydrate concentrations within honey bee flight muscle tissue and glycogen concentration within the fat body are both high during July through September (Neukirch, 1982; Shehata, et al, 1981). Flight muscles, however, have limited carbohydrate reserves which meet energy requirements for the first few minutes of flight only (Wigglesworth, 1984; Storey, 1985). When these endogenous carbohydrates are used up, exogenous carbohydrates mainly in the form of trehalose which is the principle blood sugar in most insects
is mobilized (Beenakers, et al, 1985). Haemolymph trehalose originates from glycogen stores within the fat body, the major storage area of metabolic reserves. In addition, the honey bee is also able to mobilize carbohydrates directly from its honey stomach for flight energy. When all carbohydrates become expended, the honey bee can no longer fly which is unlike migratory insects which can mobilize and oxidize lipids as a source of flight energy (Beenakkers, et al, 1969, 1985; Storey, 1985).

Classical investigations on insect longevity

Trout and Kaplan (1970) studied the effects of physical activity on longevity on "shaker" mutants of *D. melanogaster*. Shakers had abnormally high twitching and flying activity thus increasing the metabolic rate. They found that the rates of oxygen consumption, physical activity and total life span were interrelated. The average life span was found to be inversely proportional to the average metabolic rate. A honey bee is short lived during summer and its metabolic rate and physical activity are great, however, the time spent as a house bee before the onset of foraging activity appears most significant in determining honeybee longevity (Butler, 1984).

A depletion theory of aging discussed by some investigators (Rockstein, 1964; Sohal, 1976), assumes that
upon emergence, each insect has a fixed amount of useable energy. When this amount of energy is utilized, the individual dies. If the depletion theory is combined with the inverse relationship of lifespan and environmental temperature, which according to this theory, most insects show, then insects being more active at a higher environmental temperature would exhibit a rapid depletion of their usable energy.

Enzyme activity related to insect longevity

Activity of certain enzymes i.e. citrate synthase and possibly others has been shown to decrease with increasing age in honeybees (Johansson and Johansson, 1979; Harrison, 1986). Gunn and Gatehouse (1988) report that carnitine acetyltransferase, hydroxyl CoA dehydrogenase and PFK activities decline with increasing age in moths. Because enzyme activity decreases with age in honey bees (Johansson and Johansson, 1979) and winter bees have a greater lifespan than summer bees, it would seem logical for enzyme activity to be equal or higher in older winter bees than their younger summer bee counterpart. It is this hypothesis that was investigated and reported in this paper. Specific activity of PFK, a key enzyme in glycolysis was determined in winter and summer bees to see if the activity is, in fact, different or the same between the two groups. A
flight room was also constructed to test winter bees maintained on a simulated summer temperature and photoperiod. It is assumed that winter flight room bees "mimic" outside summer bees, thus it would seem reasonable for summer bees and flight room bees to exhibit similar lifespans and enzyme activity.

Energy currency, glycolysis, and role of PFK in metabolism

ATP is the energy currency between all anabolic and catabolic events occurring in the cell (Becker, 1986). In vertebrates and invertebrates alike, ATP is not stored in the cell, and its concentration in muscle is only 5mM to 7mM (Beis and Newsholme, 1975; Newsholme and Start, 1973). This quantity of ATP would be used up in less than a second during muscle contraction unless it is resynthesized (Newsholme, et al, 1978). Therefore, in order to maintain a steady-state quantity of ATP, the increase in rate of synthesis must be precisely equal to the amount of utilization, and specific mechanisms and controls to regulate the rate of ATP synthesis are required (Newsholme and Start, 1973). The many kinds of metabolic mechanisms which evolved for this precise and sensitive regulation include effects of substrates and products on enzymes which catalyze near-equilibrium reactions and/or effects of substrates of allosteric effectors on enzymes catalysing
non-equilibrium reactions (Newsholme, et al, 1978). This can occur directly or less directly by way of substrate cycles or interconversion cycles. ATP is regenerated from adenosine diphosphate (ADP) by way of oxidation of substances such as glucose, glycogen, fatty acids, and triglycerides.

Glycolysis produces pyruvate which ultimately produces adenosine triphosphate (ATP) via events in the Krebs citric acid cycle and electron transport chain (Stryer, 1981). The activity of PFK gives an indirect measure of ATP synthesis. The reaction catalyzed by PFK is removed far from equilibrium, therefore it is considered non-equilibrium and gives information about the maximum rate of operation of glycolysis in vivo (Newsholme, et al, 1978).

Kinetic control on the glycolytic rate occurs at PFK which is inhibited in most vertebrate and invertebrate cells by high levels of ATP, citrate and glycerol 1,3-phosphate. PFK is activated by AMP, \( \text{NH}_4^+ \), \( P_i \), ADP and fructose 1,6-diphosphate. However, PFK in insect flight muscle is not activated by ADP and fructose 1,6-diphosphate or inhibited by citrate (Storey, 1985).

Regulatory control of PFK has also been recently re-evaluated because of the discovery of fructose 2,6-diphosphate which is a potent activator of PFK acting at levels of less than 1ug (Hers and Schaftingen, 1982 in Storey, 1985). It has been thought that fructose

Physiologic control of metabolism in honey bees

Rates of metabolic reactions in worker honey bee muscle tissue vary under different conditions and respond in kind to physiological stresses which arise during their development, flight, and environmental changes to which the bees are exposed (Candy, 1981).

Metabolic pathways that are either anabolic (synthesizing), or catabolic (breaking down) have specific enzymes to catalize thermodynamically irreversible reactions (Bloxam, et al, 1973). Maximal enzyme activities in vitro are used to provide both quantitative and qualitative information about metabolic pathway activities (Crabtree and Newsholme, 1970). Assay of the activities of key regulatory enzymes may provide information which may be tied in with longevity of winter versus summer honey bees. Johansson and Johansson (1979), state that enzyme activity in honey bees (no specific enzymes documented) decreases with increasing bee age during summer, but remains high during winter, which according to their model, is an indicator that aging is suspended during winter months.
It was decided to assay PFK in this study because of its role as a key regulatory enzyme in glycolysis. This enzyme is irreversible and presumed to be rate limiting in aerobic metabolism along with its control over the overall rate of glucose utilization in muscle (Opie and Newsholme, 1967; Storey, 1985).

Because metabolism is complex and many factors may affect it, success in finding a difference in PFK enzyme activity in winter versus summer bees should show whether or not this enzyme could be used as an indicator in aging.

This investigation

PFK activity of flight muscle was examined in summer and winter bees to see if there was a difference. As an extension of this work, an indoor flight room maintained on a summer photoperiod and temperature was used to test bees born and raised during winter months under these conditions. I expected PFK activity in muscle tissue from flight room bees to be similar to that of summer bees.

Maximal catalytic activity of PFK in vitro catalyses a non-equilibrium reaction and approaches saturation with respect to its substrate. Similar maximum activities may be expected in vitro (Crabtree and Newsholme, 1972, 1975; Zammit and Newshome, 1976). PFK, being a regulatory enzyme for glycolysis, provides information on the maximal rate of
operation of glycolysis and thus, aerobic metabolism in **vivo** (Crabtree and Newsholme, 1972).

The portion of the metabolic pathway used, (Figure 1), in assaying PFK starts with the catalysis of fructose 6-phosphate into fructose 1,6-diphosphate by PFK. ATP serves as the phosphoryl group donor. Glyceraldehyde 3-phosphate and dihydroxyacetone phosphate are then produced from fructose 1,6-diphosphate by the enzyme aldolase. The enzyme triose phosphate isomerase maintains equilibrium between these products. 1,3-Diphosphoglycerate is then produced from the catalysis of glyceraldehyde 3-phosphate by the enzyme glyceraldehyde 3-phosphate dehydrogenase. At this step in the pathway NADH is oxidized (Stryer, 1981). The oxidation of NADH measured spectrophotometrically provided the data used in calculating the activity of PFK in **vivo**.
Figure 1. Glycolysis. The portion of the glycolytic pathway used in the assay begins with fructose 6-phosphate and ends with 3-phosphoglycerate.
Materials and Methods

Chemicals and Enzymes

All chemicals and enzymes were obtained from Sigma Chemical Co., St. Louis, Mo., U.S.A., and were used without further purification. The extraction medium used in the homogenizer contained 50mM-Tris-HCL buffer, pH 7.6, containing 5mM MgSO₄ and 1mM EDTA (after Clark, et al, 1973). Additionally, 1mM EGTA, used to chelate Ca, and 100mM-NaF were added (Storey, personal communication). The 1 ml aliquot used in the PFK assay contained 50mM-Tris-HCL buffer, pH7.6, 1mM MgSO₄, 1mM ATP, 1mM fructose 6-phosphate, 1mM dithiothreitol, 0.3i.u. aldolase, 3.6i.u. triose phosphate isomerase, 0.5i.u. glycerol phosphate dehydrogenase, and 0.2mM-NADH (Clark, et al, 1973). Additionally 0.1M NaF, 1mM EGTA, and 1mM EDTA were added (Storey, personal communication).

Sources of Honey bees

Worker-bees (Apis mellifera L.) used for outdoor winter and summer experimentation were obtained from the S.U.N.Y. Brockport apiary (43°12' N, 77°41' W), Monroe County, New York. Ten-frame Langstroth hives kept outdoors housed the queenright colonies. The single colony used in winter...
contained approximately 25,000 workers and one queen. A different colony was used in summer which contained approximately 45,000 workers and one queen.

Worker-bees used for winter testing which were maintained on a June 21st photoperiod (L:D = 16:8) were kept in an indoor flight-room (Figure 2), located in Lennon Hall on the campus of S.U.N.Y. Brockport, from November 30, 1987 until April 23, 1988. Three nucleus colonies (Dreiwabekasten, abbreviated DWK) were used in the flight room, each having six removable frames 10 cm x 10 cm. All three nucleus colonies were populated initially with about 1500 workers from an outdoor colony plus one mated queen each. Occassional addition of worker-bees from outside colonies was neccessary to maintain populations of about 1500 worker bees because colonies of honey bees do not develop as well in flight rooms as they do in nature (Van Praagh, 1987). In fact, a continual decline occured in all developmental aspects of colonies kept in a flight room (Czoppelt, 1987). Worker bees used for enzyme assays were emerged from eggs laid by flight-room queens, and they were reared in the flight-room. Sealed brood was not added.

Fluorescent tubes, fed by 60 Hz AC at 120 volts were used to provide the light source (Van Praagh, 1972; 1975). Lights were arranged horizontally on the ceiling and east and west walls providing an east-west panorama including dawn and dusk (Czoppelt, 1980; Van Praagh, 1972). The light
Figure 2. The bee flight room at the S.U.N.Y. Brockport campus, Lennon hall. L = lights; N = flight cage; F = feeding station with tray containing pollen and inverted mason jars containing sugar-water solution and water; H = bee hives (DWK).
intensity was modified to simulate a June photoperiod. Length of photoperiod was controlled by time clocks. No light fixtures were on the north and south walls. To simulate a 16 hour day including dawn and dusk, automatic switching on of light rows numbered 1 occurred at 0430 h. At 0600 h, light rows numbered 2 switched on, followed at 0730 h by light rows numbered 3. Light rows numbered 4 switched on at 0900 h, and finally at 1030 h, light rows numbered 5 switched on thereby giving full flight room daylight. Light intensity was not measured. To simulate dusk to dark, light rows switched off in reverse order starting at 1900 h until 2130 h. So that no direct light source could be seen by the bees, light reflectors faced the walls and white paper was placed between the light fixtures and flight cage to diffuse the light. Preliminary tests showed bees were attracted to direct light sources and became disoriented. Air circulated continuously through the flight room via a ceiling ventilator which removed stale air. Temperature was controlled by a thermostat and maintained at $32^\circ\pm2^\circ$C. Relative humidity was set at 60%.

Sugar-water, pollen, and fresh tap water were given to the bees daily. The sugar-water solution (55% sugar determined by a refractometer, 2.0M) was provided ad libitum in an inverted mason jar feeder. The sugar-water solution was changed daily to prevent fermentation (Kefuss, 1978). Fresh frozen pollen was dried 24 hours at $67^\circ$C in an oven,
pulverized in an electric blender and distributed evenly inside a feeding tray using a sifter. The same pollen source was used in all feedings to keep nutritional factors constant. The feeders were placed on a stand in the flight room about 2 meters from the nucleus hives. Bees were able to collect pollen by hovering above the pollen tray and periodically descending into the tray to scoop up the ground pollen grains with their legs. They then transferred the pollen to their pollen baskets (corbicullae). It was noted that the finer grains were taken preferentially and when they were gone, bees would be seen chewing the larger grains into smaller transportable pieces. Colonies were checked daily to monitor population, pollen and honey collection, occurrence of sealed and unsealed brood, general health, and sufficient empty comb for egg laying. Empty comb was continually used to replace previously empty brood rearing comb which became filled with nectar.

Experimental animals

Because of age dependent physiological functions, all worker bees used in the assay were four weeks of age or older. Previous studies have shown that summer adult bees between 16 and 25 days of age are engaged in maximal foraging activities (Harrison, 1986). Thus, at four weeks of age, adult bees will exhibit all the physiological
transformations that enhance flight capacity. Allen (1958) reported that oxygen consumption increases with age in honey bees, therefore, the oxidative capacity of flight muscles may increase as well. The increase she found in thorax-specific oxygen consumption may be due to the increase in activities of enzymes regulating the rate of carbohydrate catabolism (Harrison, 1986).

Flight room bees in this study were also taken at 4 weeks of age in the assumption that they follow a similar life pattern and span as summer bees. Winter bees used for testing were of an age greater than 4 weeks because of their longer life span. If aging is "suspended" in winter bees, it would seem logical for the PFK activity to be as high or higher in older winter bees than summer bees taken at 4 weeks of age.

Outdoor winter bees for testing were gathered on March 25th, 28th, 31st and April 1st, 6th, 7th, 12th, 13th, and 14th. The bees were removed from the outermost part of the cluster because they are usually oldest (Stephen, 1984). About twenty workers were gathered each time so that sufficient numbers were available for enzyme assays and weight determinations. Worker bees were transferred alive to the lab in a ventilated glass jar and killed within fifteen minutes by freezing at \(-80^\circ\text{C}\).

Outdoor summer worker bees were obtained by removing sealed worker-brood frames from an outdoor parent colony in
mid to late July. The frames were placed in a small observation hive kept in an incubator at $35^\circ$C and 60% RH. Each day, newly emerged workers were removed (mid to late August) and marked on the posterior thorax with enamel paint. Different colors represented different days. The same day that workers emerged and were painted, they were placed directly back into their parent colony. They were readily accepted because of their age and they presented no threat to the other bees. Worker bees identified as being four weeks of age or slightly older by 1 or 2 days from emergence, as indicated by their paint markings, were removed from the colony and transported to the lab. These bees were tested on September 12th and 13th. More bees were marked than were needed for assaying, thus, many marked bees remained in the colony. This was done so that checks could be made beyond four weeks to see if lifespans of the bees assayed were of summer duration.

Winter flightroom worker bees were obtained by first removing several sealed worker-brood frames from the three established indoor nucleus colonies on December 24th, 25th, January 30th, February 1st, 3rd, 22nd, 25th, March 13th, 15th and April 12th. The sealed brood frames were placed into a DWK which was incubated at $35^\circ$C and RH 60%. Each day newly emerged workers were marked with different colored paint and placed back in their colonies. As with summer bees, several painted bees were allowed to remain in
the flight room hives to determine life spans. Worker bees were removed four weeks later and placed in a glass jar for 15 minutes which simulated the time it took to transport winter and summer bees to the lab. They were then frozen at -80°C. Flight room bees were tested on April 6th, 19th, and 25th using bees that emerged in early-mid March.

Preparation of flight-muscle homogenate

Bees were removed from the freezer after ten minutes. While still frozen, the thorax containing the flight muscles was dissected from the rest of the bee. Five thoraxes were immediately placed in a ground-glass homogenizer kept on ice containing 2 ml of extraction medium. Preparation of flight-muscle homogenate requires the removal of the carapace also (Clark, et al, 1973), however, this requires additional time and manipulation, and not all muscle gets removed. Experimentation was conducted using control groups having flight muscle dissected from the carapace and groups not having flight muscles dissected. No difference was observed between them, thus, in all assays, the flight muscles were not dissected from the carapace.

The homogenizer, containing five thoraxes and medium, was stroked ten times, and the liquid portion was transferred by pastuer pipet to an ultracentrifugation tube. Using a pastuer pipet insured that most of the carapace
remained in the homogenizer. Following the method of Opie and Newsholme (1967), the homogenate was centrifuged for 30 minutes at 40,000 rpm at 4°C in a Beckman Ti 50 rotor. The supernatant, used for assaying, was pipetted into a small plastic storage tube, and placed immediately on ice. During initial experimentation, resuspension of the pellet showed that it contained no more PFK.

**Assay for Enzyme activities**

Enzyme assays were performed immediately after centrifugation to reduce loss of enzyme activity by inactivation (Crabtree & Newsholme, 1972). All assay solutions were prepared in advance while the homogenate was still undergoing centrifugation. Assay solutions were warmed and stored at 25°C (Crabtree and Newsholme, 1967, 1972; Newsholme, et al, 1978), by placing them in a temperature controlled water bath. For each test run, the required amount of supernatant was pipetted from its container at ice cold temperature and held in a micropipet at room temperature. Aliquots of the assay mediums (stored in the water bath) were removed as needed and immediately pipetted into a 1 ml cuvette. The supernatant was then pipetted into the cuvette also. The combined assay medium and supernatant were equilibrated by asparating the mixture once using a pastuer pipet. The cuvette was then placed
immediately in the spectrophotometer for reading. The entire procedure was completed in about 4 minutes. PFK activity was measured by the rate of oxidation of NADH in a Milton Roy Spectronic 1200 spectrophotometer at 340nm.

Readings began fifteen seconds after the cuvette was placed in the spectrophotometer and at fifteen second intervals for the next three minutes.

Standard curve

A standard curve using analytical grade PFK was used in a few assay medium mixtures to determine if PFK was, in fact, being measured. A test for linearity was also obtained by varying the concentrations of PFK.

Reagent omission

To determine that PFK was reaching its maximum reaction potential, and that the other enzymes and substrates in the reaction were not limiting, several enzymes along with ATP, NADH, and Fructose 6-phosphate were left out and the reaction was allowed to proceed. In each case, the reaction proceeded very slowly or not at all as compared to having all reagents present. This means that PFK is necessary for the reaction to proceed.
Specific activity formula for PFK

The equation used for calculating PFK specific activity arises from Beers' law (Rendina, 1971). \( A = (a)(b)(c) \).

where \( A \) = absorbance at 340 nM.
\( a \) = molar absorbtion coeff.
\( b \) = pathlength = (1.0 cm).
\( c \) = concentration.

Molar absorbtion coeff = \( 5.9 \times 10^3 \text{M}^{-1}\text{cm}^{-1} \) for
NADH at 340 nm. (Clark, et al, 1973)

Thus, \( \text{Absorbance}_{340} = (5.9 \times 10^3 \text{M}^{-1}\text{cm}^{-1})(1\text{cm})(c) \)

The oxidation velocity of NADH was obtained from the first 60 seconds of the record, and the slope was calculated by subtracting the 60 sec number from the number at 0 sec. Slope was used in the above equation as follows:

\( c = \frac{(\text{slope})}{(5.9 \times 10^3)} = X \text{ mmol/min NADH oxidized} \)
The resultant value $X$ was divided by 2 because two NADH molecules are needed for each fructose 6-phosphate molecule entering glycolysis.

$$\frac{(X \text{ mmol/min NADH oxi.})}{(2)} = X \text{ mmol/min of two NADH being oxidized, or, one F 6-P molecule.}$$

Millimoles were then changed to micromoles, thus $X$ is multiplied by 1000.

$$(X \text{ mmol/min}) \times (1000) = X \text{ umol/min}$$

Two mL = 2000uL of homogenate were used to spin down the flight muscles, and 10uL, 25uL, 50uL, 100uL, and 150uL samples were drawn from the 2000uL sample for the PFK assay. The 2000uL were divided by each assay amount to yield the fraction which was then multiplied by $X$.

$$(X \text{ umol/min}) \times \text{ (fraction number)} = X \text{ umol/min}$$
Five thoraxes were used in each 2 mL homogenate. Five sets of flight muscles were weighed from control groups brought in at the same time as test groups. Flight muscles were carefully dissected and weighed to within 0.1 mg. As the value of one bee flight muscle was wanted, average values were obtained for summer, winter, and flight room bees and were used separately for each group's calculation.

\[
\frac{(X \, \text{umol/min})}{(\text{avg wt of 5 flight muscles} = x \, \text{grams})} = X \, \text{umol/min/gram flight muscle tissue}
\]

**Bradford protein analysis**

Protein content of flight muscle for each group was determined by Bradford protein analysis (Bradshaw, 1966). Stored frozen flight muscle homogenates previously used in activity assays were placed in a styrofoam container containing crushed ice. Once the samples reached liquid state, they were gently shaken by hand to equilibrate the contents. In the Bradford assay, 200 uL of Bio-Rad reagent was combined with 800 uL of distilled water containing 5 uL and 10 uL amounts of homogenate at room temperature. A standard curve using serum bovine albumin was made to determine the unknown homogenate protein concentration (Figure 3). Absorbance was read at 595 nm on a Milton Roy Spectronic 1200 spectrophotometer. Each assay medium was prepared in the cuvette immediately prior to adding
Figure 3. Bradford protein calibration curve.
homogenate. Once the homogenate was added, the contents of the cuvette were aspirated several times with a pastuer pipet, and the cuvette was placed in the spectrophotometer and read after three minutes.

To determine the protein content of flight muscle for each group, the following calculation was used.

\[ x \text{ ug protein} = \frac{(2000uL)(uL \text{ homogenate added})(\text{conc})}{(5)} \]

Note: Original homogenate contained 2000uL. Concentration was obtained from absorbance readings using the standard curve.

Since five bees were used in the 2mL homogenate, the resultant figure was divided by five to obtain values for single bees.

Analysis of data

Individual body masses were recorded from 2 groups of 5 bees each from summer, 6 groups of 5 bees each from winter and 2 groups of 5 bees each from the flight room. These bees were obtained from the same sample of bees brought to the lab for assaying. Body mass was measured from fresh bees, and from these same bees, a net or empty mass was recorded (fresh mass minus the contents of the honey stomach and digestive tract). Empty bee mass provides a more accurate measurement than fresh mass because the contents of
a full fresh bee can vary greatly by over 20 mg (Southwick, unpublished). To obtain the net mass, a flat forcep device was used to squeeze the abdomen of each bee removing the contents. Masses of the entire thorax, and flight muscle tissue which was excised from the carapace, were also recorded. Bradford protein analysis gave results of protein amounts within the flight muscles of the three groups.

Enzyme velocities and specific activities were calculated using identical aliquots of homogenate from each group.

Analysis of variances (ANOVA) were done using MINITAB on the S.U.N.Y. Prime computer and statistical software on the MacIntosh microcomputer along with tables from Zar (1984) to determine statistically significant differences in the following sets of data for summer, winter, and flight room bees.

1. Live (fresh) body mass
2. Net body mass.
3. Thorax mass
4. Fresh flight muscle tissue mass.
5. Protein content of flight muscle tissue.
6. Enzyme velocity (rate of reaction).
7. Specific activity of PFK per unit muscle tissue.
8. Specific activity of PFK per unit muscle protein.
9. Life-spans (from marked bees).
Results

Mass of fresh body, net body, thorax and flight muscle tissue of bee.

No significant differences (P = 0.972) in fresh mass were found between summer, winter and flight room bees (Table 1). The fresh body mass averaged 115.1 ± 1.81 mg for all three groups combined (n = 50). No significant difference in mass occurred among groups within each season.

Net mass (honey stomach and digestive tract contents removed) was, of course, less than fresh body mass. No significant differences (P = 0.853) in net mass was found between summer, winter and flight room bees (Table 1). The net mass averaged 111.3 ± 1.6 mg for all three groups combined (n = 50). No significant difference in net mass occurred among groups within each season, however, an average decrease of 4 ± 0.2 mg occurred indicating G.I. tract content mass. Normalizing on net mass rather than fresh mass reduced the variation in the data by 11% (SE decreased by 0.2 mg). It was noted that although most bees lost on average 4 mg, several bees lost over 10 mg of mass between fresh and net mass measurements which is a significant difference for those bees individually. The
G.I. contents were similar in color (brown to tan) and texture (slightly viscous liquid) in all bees measured.

No significant differences ($P = 0.865$) in thorax mass occurred between summer, winter and flight room bees (Table 1). Thorax mass averaged $30.5 \pm 0.64$ mg for all groups combined ($n = 50$). The thorax mass represents approximately 26% of the fresh body mass. No thorax mass differences were noted among groups of bees within each season.

No significant differences ($P = 0.956$) were observed in flight muscle tissue mass between summer, winter and flight room bees (Table 1). Flight muscle tissue mass averaged $13.7 \pm 0.45$ mg for all groups combined ($n = 50$). No differences in mass were noted among groups within each season. Expressed as a fraction of muscle mass versus fresh body mass reveals that summer, winter and flight room bees have approximately 12% muscle tissue in their bodies (12.5% using net mass).

Protein content of flight muscle

The average protein masses in flight muscle tissue from summer, winter and flight room bees are shown in Figure 4. Winter bees have significantly less protein content than summer and flight room bees ($P = 0.046$). Protein mass averaged $1.7 \pm 0.14$ mg in winter bees, $2.3 \pm 0.20$ mg in summer bees and $2.1 \pm 0.18$ mg in flight room bees. No significant difference occurred between summer and flight room bees.
room bees. Expressed as a fraction of protein mass versus muscle mass reveals that winter bee flight muscle contains 12.4% protein, summer bee flight muscle contains 16.5% protein and flight room bee flight muscle contains 15.4% protein.

Enzyme velocity (rate of reaction) and specific activity of PFK

Enzyme velocity (rate of reaction) using the 50 µL aliquots was the same in summer, winter and flight room bees (P = 0.235). Figure 5 shows the in vitro PFK reaction rates (25 µL and 50 µL) for the three groups. The velocity, expressed in absorbance units (340 nm) per µL homogenate (50 µL aliquot values presented) shows that summer bees have a rate of 0.37 ± 0.06, winter bees (0.35 ± 0.19), and flight room bees (0.24 ± 0.06). Since the 25 µL aliquot values are only 65% of the 50 µL aliquot values, the 25 µL values are listed in the appendices only.

Figure 6 shows the specific activity of PFK per unit muscle tissue for summer (18.31 ± 3.15), winter (17.21 ± 9.28), and flight room bees (12.30 ± 3.43), expressed as umoles/min per g fresh muscle tissue at 25°C (using 50 µL aliquot values). There were no significant differences between summer, winter and flight room bees (P = 0.283).

Figure 7 shows specific activity per unit muscle protein (50 µL aliquot values presented) in summer bees (123.7 ±
29.70), winter bees (141.0 ± 76.16), and flight room bees (75.0 ± 22.50). Expressed as umole/min per g protein mass at 25°C, flight room bees are significantly lower than both summer bees (P < 0.01) and winter bees (P < 0.03).

Life spans (from marked bees)

Checks made 60 days after emergence of summer bees revealed that of 300 bees marked, only about 50 (17%) bees remained alive. This is a good indication that bees tested were, in fact, summer bees. Due to the low numbers of brood emergence in the flight room, only 5 to 10 bees on any one occasion were available for longevity measurements. Results show, however, that only a small number of bees survived beyond 7 weeks. Most of the bees died within 5-6 weeks after emergence, some earlier. No longevity measurements were performed on winter bees because it was assumed that bees within a winter colony are all over 28 days of age.
<table>
<thead>
<tr>
<th></th>
<th>N</th>
<th>Fresh body mass (mg)</th>
<th>Net body mass (mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Summer</strong></td>
<td>10</td>
<td>115.5 ± 2.51</td>
<td>110.7 ± 2.26</td>
</tr>
<tr>
<td><strong>Winter</strong></td>
<td>30</td>
<td>115.0 ± 1.54</td>
<td>112.0 ± 1.26</td>
</tr>
<tr>
<td><strong>Flight room</strong></td>
<td>10</td>
<td>114.7 ± 1.38</td>
<td>111.3 ± 1.21</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th></th>
<th>N</th>
<th>Thorax mass (mg)</th>
<th>Flight muscle mass (mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Summer</strong></td>
<td>10</td>
<td>30.2 ± 0.55</td>
<td>13.9 ± 0.45</td>
</tr>
<tr>
<td><strong>Winter</strong></td>
<td>30</td>
<td>30.9 ± 0.75</td>
<td>13.7 ± 0.38</td>
</tr>
<tr>
<td><strong>Flight room</strong></td>
<td>10</td>
<td>30.4 ± 0.61</td>
<td>13.6 ± 0.53</td>
</tr>
</tbody>
</table>

Table 1. Fresh body mass, net body mass, thorax mass and flight muscle mass of honey bee workers under three treatments. Data are presented as means ± SE, with N representing the numbers of individuals.
Figure 4. Protein mass in flight muscle tissue of summer, winter and flight room bees. The tops of the bar graph are presented as means with SE.
Figure 5. Enzyme velocity (rate of reaction) for summer, winter and flight room bees. Velocities were measured at 25 uL and 50 uL aliquots. The means and SE are presented.
Figure 6. Specific activity of PFK per unit muscle tissue for summer, winter and flight room bees. Velocities were measured at 25 uL and 50 uL aliquots. The means and SE are presented.
Figure 7. Specific activity of PFK per unit muscle protein for summer, winter and flight room bees. Velocities were measured using 25 uL and 50 uL aliquots. The means and SE are presented.
The mean body mass of the worker honey bees used in this study (115.1 ± 1.81 mg) is normal for this species. Table 2 lists a summary of fresh body mass values published by a number of investigators. Literature values for flight room bee mass have not been found. This is not likely to be a problem because there were no significant differences in mass values among the three groups in this study.

The average net body mass (contents of the honey stomach and digestive tract removed) was 111.3 ± 1.60 mg. No significant differences were observed among the three groups. Table 2 lists literature values.

<table>
<thead>
<tr>
<th>Fresh (mg)</th>
<th>Net (mg)</th>
<th>Source</th>
<th>Age (days)/season</th>
</tr>
</thead>
<tbody>
<tr>
<td>115.1 ± 1.81</td>
<td>111.3 ± 1.60</td>
<td>This study</td>
<td>&gt; 28 / all</td>
</tr>
<tr>
<td>100</td>
<td>-</td>
<td>Farenholz (1989)</td>
<td>- / ?</td>
</tr>
<tr>
<td>&lt; 100</td>
<td>-</td>
<td>Harrison (1985)</td>
<td>&gt; 30 / May-</td>
</tr>
<tr>
<td>155</td>
<td>-</td>
<td>&quot;</td>
<td>10 / July</td>
</tr>
<tr>
<td>130</td>
<td>-</td>
<td>&quot;</td>
<td>20</td>
</tr>
<tr>
<td>100</td>
<td>-</td>
<td>&quot;</td>
<td>30</td>
</tr>
<tr>
<td>70 to 150</td>
<td>-</td>
<td>Allen (1959)</td>
<td>- / ?</td>
</tr>
<tr>
<td>85 to 150</td>
<td>-</td>
<td>Rothe, et al (1989)</td>
<td>- / ?</td>
</tr>
<tr>
<td>109</td>
<td>105.0 ± 10.0</td>
<td>Altman (unpub.)</td>
<td>- / Mar.</td>
</tr>
</tbody>
</table>

Table 2. Fresh and net body mass of the honey bee. Net mass is Fresh mass minus G.I. contents.
Flight muscle tissue mass measured in this study averaged $13.7 \pm 0.45$ mg which is approximately $12\%$ fresh body mass. No significant differences were noted among the three groups. Flight muscle tissue values in this study correspond closest to those reported by Crabtree and Newsholme (1972) in which they reported that flight muscle tissue comprises $13\%$ of the fresh body mass. Southwick and Heldmaier (1987) reported that flight muscle tissue comprises $20\%$ of the fresh body mass while Nachtigal, et al (1989) reported it to be $15\%$.

Thorax mass from this study averaged $30.5 \pm 0.64$ mg. Harrison (1985) reported honey bee thorax values of $27.4$ mg for May and July, and that the thorax mass does not change with increasing age.

The protein content of the flight muscle tissue from flight room bees ($2.05 \pm 0.18$ mg) was not significantly different from that of summer bees ($2.27 \pm 0.2$ mg). The protein content from winter bees ($1.67 \pm 0.14$ mg) was, however, significantly lower than both groups. Southwick (unpublished), studying *Apis mellifera carnica* in Germany reported that the average flight muscle protein mass from outdoor bees in January was $7.79$ mg, May ($6.89$ mg) and August ($7.36$ mg). The protein mass values from this study for all three groups are much lower, however, the winter mass value in this study is proportionally lower similar to
that found by Southwick. Winter bees overwinter on stored pollen, thus a nutritional factor may have caused the protein content to be lower in these bees. It may also be possible that the low winter protein value was the result of incomplete homogenization of the protein which resulted in partially insolublized protein which ended up in the pellet after centrifugation.

Flight room bees which were selected for longevity studies did not live beyond 60 days and were found to have lived a shorter time than their outdoor winter counterparts. However, flight room bee life spans were very similar in duration to the summer bee life spans. Perhaps the reason for the relatively short life spans of flight room bees was the result of them becoming disoriented within the flight cage and unable to return to their hive, or for some other reason associated with flight room conditions. Though flight room bees lived about the same duration as summer bees measured in this study, caution is suggested in assuming that winter flight room bees become, in essence, outdoor summer bees.

For the in vitro PFK enzyme reaction experiments, it was found that values obtained at 25 uL and especially the 50 uL aliquots were best for use in this study. When 10 uL aliquots of homogenate were used, an extremely slow rate of reaction occurred (less than 0.005). It may be that the
homogenate was too diluted in the assay medium to work optimally. Concomitantly, when 100 \mu L aliquots were attempted, an extremely rapid rate of reaction occurred (greater than 0.5) which led to substrate depletion too quickly for slope calculations to be made. In enzyme kinetics, as the homogenate is doubled, the rate of the reaction should also double. This did not happen, the rate of the reaction increased by a multiple of five when proceeding from 25 \mu L to 50 \mu L aliquots (Figure 6). The rate should have increased by a multiple of only two. Therefore, the 25 \mu L aliquot values for the specific activity of PFK measurements, derived from the reaction rate, are only 65% of the 50 \mu L aliquot values.

The results of the specific activity of PFK (expressed as umol/min per g fresh mass at 25^{\circ}C) from this study (50 \mu L aliquots) for summer bees averaged 18.3 \pm 1.29. Outdoor winter bees averaged 17.2 \pm 2.13, and flight room bees averaged 12.3 \pm 1.30, substantially less than that of un-restrained animals. Crabtree and Newsholme (1967, 1972) and Newsholme, et al (1978) reported the specific activity of PFK in honeybee flight muscle tissue to be 20.0 \pm 2.0 umol/min per g fresh mass at 25^{\circ}C. Seasonal effects and diet on the specific activity of PFK were not included in their study.

When contrasting the protein content of the flight muscle from the three groups with their corresponding
specific activity of PFK values, it was observed that even though there was no significant difference in the protein content between summer or flight room bees (Figure 4), flight room bees had a significantly smaller value for specific activity per unit muscle protein than both summer and winter bees (Figure 7). Perhaps the differences in muscle protein and specific activity are related to thermoregulation by winter bees whereby they shiver their flight muscles and thus may not need to express the full flight potential that a summer bee would. However, the fact that winter bee protein is less than summer and especially flight room bee protein is interesting and may be due to the before mentioned discussion. More measurements of muscle protein from winter bees should be made before any conclusions are drawn.

Further possibilities concerning the difference between the three groups is that brood rearing is generally non-existant during winter whereas brood rearing did occur in the flight room (infrequent) and during summer (extensive). This may in turn affect hormone levels thus affecting the metabolic rate and PFK levels. It is possible at the time winter bees were tested in this study, brood rearing had begun in their colonies which may have changed their metabolism and PFK values. Also, although winter bees were gathered in accordance to literature suggestions for gathering overwintering bees in early spring, if brood
rearing had begun there was certainly no guarantee that young bees were not gathered. Perhaps due to a few basic parameters such as flight by summer bees, lack of significant flight by flight room and winter bees, brood rearing, thermoregulating and the fact that flight room bees are not in a natural environment may cause events such as hormonal growth regulators to be influenced. The affect of these regulators may be increased or decreased gene transcription of various protein products including isozymes, muscle protein, allosteric effectors and other hormones affecting metabolism and ultimately the longevity of the bee. Extensive flight and extensive brood rearing was absent in flight room bees which may have led to a decrease in the synthesis of PFK. Even though diet, lighting, temperature etc... were tightly controlled, it was observed that events which would occur in the wild such as intensive brood rearing and flight did not occur to the same extent within the flight room. It is therefore safe to conclude that flight room bees were not in a "perfect" summer environment.

Correlations have been found between the activity patterns of several enzymes and the development and decline of flight activity in a number of insects which utilize carbohydrates as energy for flight (Beenakers, et al, 1975). For example, the rise and fall of flight activity in Phomia...
regina, *P. terrae novae* and *D. melanogaster* can be reflected in an identical rise and fall of thoracic Glyceraldehyde dehydrogenase activity (Baker, 1975). Perhaps a similar trend occurred in the specific activity of PFK from flight room bees because of their very short and infrequent flights seen in this study. They were engaged in some flight activity and had access to proper nutrition which may account for their muscle mass and protein levels being the same as summer bees, however, the distance they traveled to forage was only 2 meters. It was noted that the number of foragers dropped dramatically once the food source was discovered and exploited. Thus, a very small percent of the flight room bees were even engaged in flight. Other bees seen outside their colonies were either involved in short flights to defecate, or hanging on the flight room net. In addition, the flight room bees were not required to maintain the internal colony temperatures as did their outdoor winter counterparts. Thus, it may be that the PFK activity in the flight room bees was not at its full potential unlike outdoor summer bees engaged in lengthy flights or winter bees shivering their flight muscles to produce heat for their colony.

Based on the fact that a decline in several enzyme activities occurs with increasing age in honeybees and other insects (Gunn and Gatehouse, 1979; Harrison, 1986; Johannson
and Johannson, 1979), the fact that there was no difference in the specific activity between summer bees and winter bees is interesting. The enzyme activity should decrease with increasing bee age, but the specific activity in winter bees assumed older than 120 days was not significantly different from activity in summer bees aged no more than 30 days. Thus, the PFK activity may indicate that aging is suspended in winter.

Carbohydrates are used as a major source of metabolic fuel in winter bees and appear to be supported by the results of this study in part due to the specific activity of PFK in winter bees not being different than summer bees. Shehata, et al, (1981) found high consumption of exogenous carbohydrates in winter bees which is necessary for cluster temperature regulation.

At one point in the summer study, a group of marked bees was obtained on the outside of the hive after returning from flight. When the homogenates of these bees were run in the spectrophotometer, the reaction rate proceeded extremely slowly for the first 10 to 50 seconds. The reaction appeared to then reverse its "course" resulting in a negative rather than a positive slope. It may be that there was ATPase activity present in this group related to recent flight. Bees used in this study were gathered from inside the colonies and did not appear to have been engaged in
recent flight. Therefore, it is important for future testing of bees to be aware of the extremely different results which can be obtained as discovered by accident in this study.

Classical studies done concerning lifespans of houseflies by Ragland and Sohal (1973), where one group of flies was restricted from flight and a second group was allowed flight revealed that the flight restricted flies lived twice as long as their counterparts. Even though the flight per say may or may not directly relate to the longevity of the insect, but rather hormonal or gene regulation etc..., further investigation involving winter flight room bees could be done by restricting one group from flight and a second group being allowed to forage. Brood rearing would be used as a third independant variable. Taking the specific activity of PFK at the same chronological time from different experimental bees may yield useful information about longevity on a molecular level. Similar experimentation would involve testing summer bees allowed and not allowed flight. In this way, further information may be gathered concerning whether a winter bee is physiologically, genomically, and/or environmentally different from a summer bee. Sohal (1976), has described a hypothesis whereby developmental changes occur by selective depression of genes which is brought about by genomic
interactions with the environment. Clark (1964), states that lifespan and the rate of ontogenetic changes are strongly influenced by the environment and genetic determinants. It may also be that specific cell alterations (e.g. lipofuscin in humans) may alter lifespan. Sheldahl and Tappel (1974), have found lipofuscin-ceroid amounts greater in *D. melanogaster* at 26.7°C than at 22.0°C. It is possible that these cell alterations may influence enzymatic pathways, thus it would be interesting to experiment with bees at different temperatures and determine any possible correlation with their metabolism by testing the activity of PFK.

Sohal (1976) states that the rate of living theory and threshold theory (aging and dying phase) attempt to relate environmental temperature to longevity in poikiotherms, but he feels that a major question still concerns the relationship of metabolism and life span. It is assumed that metabolic rates vary relative to changing temperatures, but there is no evidence to support that all poikiotherms have metabolic compensation abilities. Depending on species-specific behavior and environmental conditions, physical activity may vary or remain unaffected. He states that at different ambient temperatures there are variable physiological responses which are demonstrative in poikiotherms. Thus, aging theories should take into account factors such as thermal kinetics of enzymes, isozyme
proportions, cofactors, enzyme synthesis and degradation and membrane permeabilities (Sohal, 1976).

Possible sources of error

Due to equipment failure, winter bees and flight room bees were obtained in late March and early April, rather than mid winter. However, due to low temperatures continuing during these months, outdoor winter bees remained in a relatively tight cluster which facilitated the removal of the oldest bees which are found on the outer most portion of the cluster (Stephen, 1984). Because wintering bees are born throughout Autumn, it can be assumed that the bees used in the winter study from the outdoor colonies were post-wintering adults as described by Sakagami and Fukuda (1968). Also, because egg laying stops between November and January and does not resume again until February (Shehata, et al, 1981), the probability that winter bees were, in fact, gathered for the study is good.

The population in the outdoor colonies during summer and winter appeared to follow normal growths and declines in worker bee population. However, in the flight room, worker bees from outdoor colonies had to be periodically added to insure healthy maintenance of the sealed and unsealed brood present. Abnormal declines in flight room bee populations have been reported in the literature (Czoppelt, 1987; Van
Praagh, 1987). Although both sealed and unsealed brood were present in the flight room colonies, the amount was not consistent, and may very well have induced endocrine changes which in turn may have influenced enzyme levels (Rutz, et al, 1974, 1976).

It may be the way in which food reaches the colonies in a flight room that accounts for the inconsistency of brood-rearing noted in this study (Van Praagh, 1972). There were, however, sufficient nectar and pollen stores within each colony. Van Praagh (1982) suggested that the colonies in a flight room may misinterpret the actual protein amount. This may in turn lead to cannibalism of larvae by worker bees which results from a lack of proper protein intake (Woyke, 1977). It appeared that cannibalism occurred in the flight room colonies. A high air humidity in a flight room has also been found to decrease the amount of capped brood by as much as 50% (Van Praagh, 1987). However, the relative humidity was set at 60% in this study which was between Czoppelt's suggested 45% RH and Van Praagh's 80% RH (Czoppelt, 1980; Van Praagh, 1975).

Summary

This paper was done to further explore and possibly reveal more information associated with longevity in honey bees. Perhaps a question pertaining to why honey bees in
winter live as long as they do and why summer bees are short lived is also important. Why should a summer bee live such a short time? It would make sense for a summer bee to live as long as a winter bee so that it may forage for a longer period. In addition, the queen would not need to produce such large numbers of offspring as she does. Perhaps this is the "cost" of efficient survival for these insects.

Southwick (1987) discussed the honeybee colony as being a "superorganism." This may also imply that the whole is greater than its constituents. In the same way that animals continually "turn over" cells to maintain homeostasis, a honeybee colony turns over its members to maintain a healthy state. Although costly in terms of nutrition and metabolism for its members in a summer colony, continual brood rearing and resultant early death of workers would provide trade-offs. Disease, for example, would have a harder time taking hold in a colony because infected bees with their short life spans would not continue in the colony. By continually manufacturing brood, a buffer is provided in the event of large losses of bees which could occur, for example, from a predator or insecticides. Fukuda and Sekiguchi (1966) state that expansions in colony size are at the sacrifice of individual life spans. Efficiency may also decrease with increasing bee age which would seem logical in a summer bee which expresses a large metabolic potential. If this is so, than a continual supply of new bees would
overcome this problem. The continued production of new members which occurs in honeybee colonies combined with potentially longer lived summer bees may create in time an overpopulation problem which would have too many of its members competing for the same niche. Quality of their survival would then decrease.

Long lived winter bees are essential for the survival of these insects until the following spring. If summer brood rearing patterns continued into winter, food supplies would become exhausted before the spring nectar flow. Thus, if costly brood rearing is to be discontinued for the sake of the food supply, something else must compensate. The trade-off would be in having bees going into the winter season survive into spring to re-establish the summer colony. Jeffree (1959 in McLellan, 1978) stated that an increase in population above 20,000 workers in a overwintering colony would lead to overheating. This provides an additional reason as to why brood rearing stops in winter. Free (1966), Jeffree (1955), Haydak (1957), Neukirch (1982), Ribbands (1952) and Rockstein (1964) have shown that brood rearing has large effects on honey bee life span. Studies by Fukuda and Sekiguchi (1966) have shown that longevity shortened in response to expansion of brood area and vice versa. This expansion of brood area results in more intensive brood rearing performed by existing members of the colony (Maurizio, 1950). Brood rearing also
indirectly affects foraging because the number of young foragers increases in response to food demands which results in shorter life spans (Fukuda and Sekiguchi, 1966). It may be that brood rearing is the built-in governor for longevity in honeybees. Although brood rearing can be controlled by workers, it may serve to "inform" the colony when extended life spans are needed of the workers in order to re-establish dwindling populations. Concomitantly, when sufficient numbers of bees are obtained, worker bee life spans decrease owing possibly to increased brood rearing.

Avitabile (1978) suggests that the summer and winter solstices set the biological clock for the reproductive schedule of honeybees. Shehata, et al., (1981) suggests that photoperiod, and to a lesser extent, decreasing temperatures play a role in brood rearing cessation and commencement.

In conclusion, the honeybee colony which exists perennially without the need to diapause, might seem to owe much of its success throughout much of the world to its "ability" to regulate lifespans of its workers. But why should the honey bee not diapause? Clearly those insects that diapause require very little in the way of energy cost. Honey bees expend great amounts of energy and consume large amounts of food to survive the winter freeze. Mother nature is by "nature" stingy and the way in which these insects overwinter is an exorbitant waste of energy and food and it
really doesn't make good economic sense for them to do this. Perhaps then, thermoregulation by honey bees is the result of adaptation and selective breeding and the subsequent proliferation of a specific gene allowing over a millenium entire populations of honey bees to overwinter. This proliferation of honey bees into temperate zones may have been in response to having to leave over-exploited niches and competition found in tropical areas (Roubik, 1989). The problem may have been that these bees never had the gene capability to produce "antifreeze" such as diapausing insects do. It would seem that honey bees may have already had the capacity for social existence long before they could thermoregulate, evident by a complex system of hierarchy and organization which by chance (mutation or adaptation) seems unlikely. Thermoregulation on the other hand appears more of an adaptation where they simply use their existing capabilities to keep warm. The perplexing question about overwintering, however, is the extended lifespans that honey bees exhibit. Many researchers favor the idea that the absence of brood rearing extends honey bee lifespans. Perhaps the lack of brood rearing which occurs in winter is simply a natural response that honey bees have always possessed when exogenous food supplies drop, which occur in northern climates in late fall. The resultant decline in brood rearing may cause hormonal regulators, i.e., juvenile hormone, to be decreased thus suspending the aging process.
Once spring arrives, food becomes plentiful and the natural instinct for these bees is to proliferate, and brood rearing commences causing the bees to age owing possibly to hormonal effects. This model would also need to include the honey bees' ability to store food beyond what it needs to survive daily. Perhaps before any migration into temperate regions occurred, some species stored food in this manner and others didn't. Perhaps many of these species attempted to migrate into the temperate zone, but only those which had the trait for storing food, along with other adaptations in their genome were able to survive extended winters in the temperate zone. Thus, even though it appears a waste of energy and food for these insects to overwinter this way, it may, in fact, insure their survival.
Bibliography


Corkins, C. L. 1932. The temperature relationship of the honeybee cluster under controlled temperature conditions. J. Econ. Ent. 25: 820-825.


Appendix A. Analysis of variance for total fresh body mass of bees.

<table>
<thead>
<tr>
<th>Group</th>
<th>N</th>
<th>Mean (mg)</th>
<th>StDev. (mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Winter Bees</td>
<td>30</td>
<td>115.00</td>
<td>8.45</td>
</tr>
<tr>
<td>Summer Bees</td>
<td>10</td>
<td>114.67</td>
<td>4.37</td>
</tr>
<tr>
<td>Flight room Bees</td>
<td>10</td>
<td>115.48</td>
<td>7.94</td>
</tr>
</tbody>
</table>

Pooled StDev = 7.74

Individual 95 Pct Ci's for mean based on pooled StDev.

\[
\begin{align*}
111.0 & \quad 114.0 & \quad 117.0 & \quad 120.0 \\
\end{align*}
\]

Analysis of variance (StDev.)

<table>
<thead>
<tr>
<th>Source</th>
<th>DF</th>
<th>SS</th>
<th>MS</th>
<th>F</th>
<th>P</th>
</tr>
</thead>
<tbody>
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<td>Factor</td>
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<td>3.4</td>
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<td>0.972</td>
</tr>
<tr>
<td>Error</td>
<td>47</td>
<td>2812.5</td>
<td>59.8</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>49</td>
<td>2815.8</td>
<td></td>
<td></td>
<td></td>
</tr>
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Appendix B. Analysis of variance for net mass of bees.

<table>
<thead>
<tr>
<th>Group</th>
<th>N</th>
<th>Mean (mg)</th>
<th>StDev. (mg)</th>
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</thead>
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<tr>
<td>Winter Bees</td>
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<td>111.98</td>
<td>6.92</td>
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<td>10</td>
<td>111.27</td>
<td>3.85</td>
</tr>
<tr>
<td>Flight room Bees</td>
<td>10</td>
<td>110.70</td>
<td>7.17</td>
</tr>
</tbody>
</table>

Pooled StDev. = 6.50

Individual 95 Pot Ci's for mean based on pooled StDev.

---+---------+---------+---------+--(---------·--------) (---------------·----------------) (----------------·---------------) ----+---------+---------+---------+--107.5 110.0 112.5 115.0 (mg)

Analysis of variance (StDev.)

<table>
<thead>
<tr>
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<th>F</th>
<th>P</th>
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<td>0.865</td>
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<td>Error</td>
<td>47</td>
<td>397.51</td>
<td>8.46</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>49</td>
<td>399.96</td>
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</tbody>
</table>
**Appendix C. Analysis of variance for thorax mass of bees.**

<table>
<thead>
<tr>
<th>Group</th>
<th>N</th>
<th>Mean (mg)</th>
<th>StDev. (mg)</th>
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</thead>
<tbody>
<tr>
<td>Winter bees</td>
<td>30</td>
<td>30.40</td>
<td>3.323</td>
</tr>
<tr>
<td>Summer bees</td>
<td>10</td>
<td>30.17</td>
<td>1.742</td>
</tr>
<tr>
<td>Flight room bees</td>
<td>10</td>
<td>30.85</td>
<td>2.358</td>
</tr>
</tbody>
</table>

Pooled StDev. = 2.908

Individual 95 Pct CI's for mean based on pooled StDev.

```
28.8 30.0 31.2 32.4 (mg)
```

**Analysis of variance (StDev.)**

<table>
<thead>
<tr>
<th>Source</th>
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<th>MS</th>
<th>F</th>
<th>P</th>
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</thead>
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<tr>
<td>Factor</td>
<td>2</td>
<td>2.46</td>
<td>1.23</td>
<td>0.15</td>
<td>0.865</td>
</tr>
<tr>
<td>Error</td>
<td>47</td>
<td>397.52</td>
<td>8.46</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>49</td>
<td>399.96</td>
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<td></td>
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</table>
Appendix D. Analysis of variance for flight muscle tissue mass of bees.

<table>
<thead>
<tr>
<th>Group</th>
<th>N</th>
<th>Mean (mg)</th>
<th>StDev. (mg)</th>
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<tbody>
<tr>
<td>Winter Bees</td>
<td>30</td>
<td>13.690</td>
<td>2.084</td>
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<tr>
<td>Summer Bees</td>
<td>10</td>
<td>13.600</td>
<td>1.696</td>
</tr>
<tr>
<td>Flight room Bees</td>
<td>10</td>
<td>13.850</td>
<td>1.415</td>
</tr>
</tbody>
</table>

Pooled StDev. = 1.901

Individual 95 Pct Ci's for mean based on pooled StDev.

\[
\begin{align*}
12.80 & \quad 13.60 & \quad 14.40 & \quad 15.20 \\
\end{align*}
\]

Analysis of variance (StDev.)

<table>
<thead>
<tr>
<th>Source</th>
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<th>P</th>
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<tbody>
<tr>
<td>Factor</td>
<td>2</td>
<td>0.33</td>
<td>0.16</td>
<td>0.05</td>
<td>0.956</td>
</tr>
<tr>
<td>Error</td>
<td>47</td>
<td>169.85</td>
<td>3.61</td>
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</tr>
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<td>Total</td>
<td>49</td>
<td>170.18</td>
<td></td>
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</table>
Appendix E. Analysis of variance for protein content in flight muscle of bees.

<table>
<thead>
<tr>
<th>Group</th>
<th>N</th>
<th>Mean (mg)</th>
<th>StDev. (mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Winter Bees</td>
<td>18</td>
<td>1.6727</td>
<td>0.5833</td>
</tr>
<tr>
<td>Summer Bees</td>
<td>21</td>
<td>2.2766</td>
<td>0.8975</td>
</tr>
<tr>
<td>Flight room Bees</td>
<td>12</td>
<td>2.0522</td>
<td>0.6082</td>
</tr>
</tbody>
</table>

Pooled StDev. = 0.7354

Individual 95 Pct Ci's for mean based on pooled StDev.

---+---------+---------+---------+--(---------*---------) (--------*--------) (------------·-----------) ---+---------+---------+---------+--1.40

1.75 2.10 2.45 (mg)

Analysis of variance (StDev.)

<table>
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<tr>
<th>Source</th>
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<th>P</th>
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<tbody>
<tr>
<td>Factor</td>
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<td>3.562</td>
<td>1.781</td>
<td>3.29</td>
<td>0.046</td>
</tr>
<tr>
<td>Error</td>
<td>48</td>
<td>25.961</td>
<td>0.541</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>50</td>
<td>29.523</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Appendix F. Analysis of variance for PFK enzyme velocity (rate of reaction) in honey bee flight muscle tissue.

50 uL homogenate. (absorbance at 340 nm/uL homogenate)

<table>
<thead>
<tr>
<th>Group</th>
<th>N</th>
<th>Mean</th>
<th>StDev.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Winter Bees</td>
<td>19</td>
<td>0.3473</td>
<td>0.1872</td>
</tr>
<tr>
<td>Summer Bees</td>
<td>6</td>
<td>0.3730</td>
<td>0.0646</td>
</tr>
<tr>
<td>Flight room Bees</td>
<td>7</td>
<td>0.2416</td>
<td>0.0694</td>
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</tbody>
</table>

Pooled StDev. = 0.1532

Individual 95 Pct Ci's for mean based on pooled StDev.

-----------+---------+---------+------(-----*-----)
(---------·---------) (----------·----------) ----------+---------+----~----+------0.24 0.36 0.48
(Absorbance at 340 nm)

Analysis of variance (StDev.)

<table>
<thead>
<tr>
<th>Source</th>
<th>DF</th>
<th>SS</th>
<th>MS</th>
<th>F</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Factor</td>
<td>2</td>
<td>0.0715</td>
<td>0.0357</td>
<td>1.52</td>
<td>0.235</td>
</tr>
<tr>
<td>Error</td>
<td>29</td>
<td>0.6804</td>
<td>0.0235</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>31</td>
<td>0.7519</td>
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<td></td>
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</tbody>
</table>
Appendix G. Analysis of variance for Specific activity of PFK per unit muscle tissue in honey bees.

25 uL of homogenate (umol/min per g fresh mass at 25°C)

<table>
<thead>
<tr>
<th>Group</th>
<th>N</th>
<th>Mean</th>
<th>StDev.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Winter Bees</td>
<td>13</td>
<td>11.893</td>
<td>7.680</td>
</tr>
<tr>
<td>Summer Bees</td>
<td>6</td>
<td>13.168</td>
<td>6.636</td>
</tr>
<tr>
<td>Flight room Bees</td>
<td>7</td>
<td>6.561</td>
<td>2.130</td>
</tr>
</tbody>
</table>

Pooled StDev. = 6.385

Individual 95 Pct Ci's for mean based on pooled StDev.

---+--------+--------+--------
| 5.0  | 10.0   | 15.0   |
---+--------+--------+--------
(Absorbance at 340 nm)

Analysis of variance (StDev.)

<table>
<thead>
<tr>
<th>Source</th>
<th>DT</th>
<th>SS</th>
<th>MS</th>
<th>F</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Factor</td>
<td>2</td>
<td>174.9</td>
<td>87.4</td>
<td>2.15</td>
<td>0.140</td>
</tr>
<tr>
<td>Error</td>
<td>23</td>
<td>937.5</td>
<td>40.8</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>25</td>
<td>1112.4</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Appendix H. Analysis of variance for Specific activity of PFK per unit muscle tissue in honey bees.

50 uL homogenate. (umol/min per g fresh mass at 25°C)

<table>
<thead>
<tr>
<th>Group</th>
<th>N</th>
<th>Mean</th>
<th>StDev.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Winter Bees</td>
<td>19</td>
<td>17.205</td>
<td>9.279</td>
</tr>
<tr>
<td>Summer Bees</td>
<td>6</td>
<td>18.307</td>
<td>3.154</td>
</tr>
<tr>
<td>Flight room Bees</td>
<td>7</td>
<td>12.299</td>
<td>3.434</td>
</tr>
</tbody>
</table>

Pooled StDev. = 7.589

Individual Pct Ci's for mean based on pooled StDev.

--------+---------+---------+--------
          | 10.0    | 15.0    | 20.0   |
--------+---------+---------+--------

(Absorbance at 340 nm)

Analysis of variance (StDev.)

<table>
<thead>
<tr>
<th>Source</th>
<th>DF</th>
<th>SS</th>
<th>MS</th>
<th>F</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Factor</td>
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<td>151.8</td>
<td>75.9</td>
<td>1.32</td>
<td>0.283</td>
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<td>Error</td>
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<td>57.6</td>
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</table>
Appendix I. Specific activity of PFK per unit muscle protein in honey bees.

25 uL homogenate. (umol/min per g protein mass at 25°C)

<table>
<thead>
<tr>
<th>Group</th>
<th>N</th>
<th>Mean</th>
<th>StDev.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Winter Bees</td>
<td>19</td>
<td>97.3</td>
<td>52.64</td>
</tr>
<tr>
<td>Summer Bees</td>
<td>6</td>
<td>89.1</td>
<td>48.04</td>
</tr>
<tr>
<td>Flight room Bees</td>
<td>7</td>
<td>40.0</td>
<td>21.60</td>
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</table>

Appendix J. Specific activity of PFK per unit muscle protein in honey bees.

50 uL homogenate. (umol/min per g protein mass at 25°C)

<table>
<thead>
<tr>
<th>Group</th>
<th>N</th>
<th>Mean</th>
<th>StDev.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Winter Bees</td>
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<td>141.0</td>
<td>76.16</td>
</tr>
<tr>
<td>Summer Bees</td>
<td>6</td>
<td>123.7</td>
<td>29.70</td>
</tr>
<tr>
<td>Flight room Bees</td>
<td>7</td>
<td>75.0</td>
<td>20.95</td>
</tr>
</tbody>
</table>