The Effects of Miticides on the Reproductive Physiology of Honey Bee (*Apis mellifera* L.) Queens and Drones

Lisa Marie Burley

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Advisory Committee:
Dr. Richard D. Fell, Chair
Dr. Carlyle C. Brewster
Dr. Donald E. Mullins
Dr. Richard G. Saacke

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Abstract

The effects of miticides on the reproductive physiology of queens and drones were examined. The first study examined the effects of Apistan® (fluvalinate), Check Mite+ (coumaphos), and Apilife VAR® (74% thymol) on sperm production and viability in drones. Drones from colonies treated with each miticide were collected at sexual maturity. Sperm production was determined by counting the number of sperm in the seminal vesicles. Sperm for viability assays was analyzed by dual fluorescent staining. Apilife VAR® and coumaphos significantly lowered (P<0.0001) sperm production and coumaphos treatments caused a significant decrease (P<0.0001) in the sperm viability.

The effects of miticides on queens was examined by treating queen-rearing colonies and examining the number and viability of sperm in the spermathecae of newly mated queens. Queens from each treatment group were collected after mating and the spermathecae were removed and analyzed. Colonies treated with coumaphos failed to provide viable queens and were excluded. Apilife VAR® was found to significantly decrease (P<0.0016) sperm viability. No significant differences in sperm numbers were found between treatments.

The effect of miticides on sperm viability over time was also examined. Drones were reared as described, but the spermatozoa were collected as pooled samples from groups of drones. The pooled samples from each treatment were subdivided and analyzed periods of up to 6 weeks. Random samples were taken from each treatment (n = 6 pools) over a period of 6 weeks. The exposure of drones to coumaphos during development significantly reduced sperm viability for all 6 weeks, and caused a large decline in week 6. The potential impacts of these results on queen performance and failure are discussed.

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1. Introduction

Interactions between humans and honey bees date back at least 9000 years and are shown in prehistoric cave paintings depicting people on ladders with baskets collecting honey from nests on a rock cliff (Caron 1999). This early interest in hive products led to the development of man-made hives and beekeeping practices for the efficient management of honey bee colonies. The development of modern hives also led to an increased interest in the study of honey bees as a biological organism. Over the years researchers have increased our understanding of the biology and behavior of the honey bees, from how bees navigate, to the inner workings of their complex social behavior. Research has also led to improvements in management practices. However, problems continue to plague the beekeeping industry, particularly those associated with colony health. Researchers and beekeepers, for example, have failed to find methods to safely control the parasitic mites affecting honey bees in the United States and elsewhere in world. The introduction of tracheal mites to the United States in 1984 and the varroa mite in 1987 caused populations of both feral and managed colonies to plummet (Ottis and Scott-Dupree 1992, Kraus and Page 1995, Finley et al. 1996, DeJong 1997, Wilson et al. 1997, Hunt 1998, Sammataro et al. 2000). Even today colonies in the United States face serious problems from the varroa mite, as well as new problems from the mysterious Colony Collapse Disorder.

The most common methods for parasitic mite control in the United States have been the use of synthetic chemical miticides placed directly in honey bee colonies. More natural products, such as organic acids and plant-derived oils, have also made their way into the U.S. market. However, despite the use of these chemicals, colony numbers have continued to decline. In addition, other problems have emerged from the use of miticides in colonies, including the accumulation of chemical residues in beeswax, as well as mite resistance to the chemicals. Concomitant with this miticide use, beekeepers have faced increased problems with maintaining productive queens in their hives. Problems such as queen failure, heightened queen supersedure rates, and the inability of hives to re-queen themselves have been reported (Sanford 2001). Thus, the current study on the effects of miticides on queen and drone honey bees was conducted to better understand the possible effects miticides could have on the reproductive physiology of drone and queens. A better

understanding of the chronic impacts of miticides provide insights into possible causes for the current problems associated with queen functionality in the hive, and thus hive health and productivity.

This study consists of three major parts. The first part involves a study of the effects of miticides on the reproductive physiology of drones with regard to spermatozoal production and viability. The second part involves an examination of the effects of miticide exposure on queens during development, and how they might impact mating success and the number and viability of sperm in the spermathecae. The third part involves an *in vitro* study on the latent effects of miticide exposure on sperm viability over time in drones exposed to treatments during development and maturation.

2. Review of Literature

2.1 Parasitic Mites of Honey Bees

Mites that parasitize honey bees have become a global problem that threatens the survival of both managed and feral honey bee colonies, as well as agricultural crops that depend on pollination. *Acarapis woodi*, the tracheal mite, was first discovered in England in 1921 (De Jong et al. 1982a), and then later in the United States in 1984 (Shimanuki et al. 1994). *Varroa destructor* (Anderson and Trueman), the varroa mite pest originally of *Apis cerana*, the Eastern Honey bee, was discovered in the United States shortly after the tracheal mite in 1987 (Shimanuki et al. 1994, Needham 1998). The introduction of *A. woodi* led to increased colony losses within two years; it took four or five years before significant losses could be attributed to *V. destructor*. Bee mites have a greater potential for dispersal than most other Acari due to the movement of hives for agricultural pollination, and by the movement of the bees themselves by swarming and drifting behaviors (Sammataro et al. 2000). Mite presence in a hive can induce stress that can increase susceptibility to diseases such as nosema and chalkbrood that might not have been apparent before the mite infestation (Finley et al. 1996). Tracheal and *Varroa* mites are major contributing factors to the loss of honey bee colonies today (Finley et al. 1996). Since their introduction to the U.S., the impact of the mites have changed the pest management systems of beekeepers and devastated feral and commercial honey bee populations (Shimanuki et al. 1994).

Tracheal mites live and reproduce within the abdominal air sacs and trachea of the prothoratic spiracles of an adult bee's respiratory track (De Jong et al. 1982a, Henderson and Morse 1997, Sammataro et al. 2000). Egg, larvae, and adult mites are all found in the honey bee respiratory system composed of ten spiracles and branched tracheal tubes (Henderson and Morse 1997). Tracheae of infested honey bees will show brown blotches and brown scabs, or even appear entirely black, depending on the intensity of the infestation (Delfinado-Baker 1984). There has been much speculation on what damage tracheal mites cause to honey bee individuals, including mechanical obstruction of the airways and flight impairment because of reduced oxygen supply to flight muscles (Henderson and Morse 1997). However, Gary and Page (1989) did not find any difference in the number of foraging trips, frequency of trips, time between foraging trips,

and frequency of pollen collection between tracheal mite infested honey bees and non-infested bees. The amount of nectar a bee brings back to the colony also is not hindered by tracheal mite infestation of foraging bees (Gary and Page 1989).

Varroa mites have a reddish-brown coloration and their bodies are dorsoventrally flattened to fit ideally under the abdominal segments of the bee for feeding on the hemolymph by piercing the intersegmental membranes with their chelicerate mouthparts (De Jong 1997). Varroa mites are ectoparasites that feed on the hemolymph of the late larval and pupa stages of bee brood. However, female mites are more likely found feeding on the hemolymph of adult bees which they use as short-term hosts for dispersal (Sammataro et al. 2000). Damage to individual bees by varroa mites can be extensive. Schneider (1986) found reduced flight frequencies in drones infested with varroa. De Jong et al. (1982b) reported a 6-25% loss in body weight of workers depending on infestation levels. The life span of adult honey bees are reduced 34-68% due to varroa infestation (De Jong and De Jong 1983). The feeding activities of varroa also cause a 15-50% loss of haemolymph protein content and total haemolymph volume in emerging bees (Smirnov 1978, Weinberg and Madel 1985).

The loss of colonies to tracheal and varroa mites has been widely reported (De Jong 1997, Wilson et al. 1997, Sammataro et al. 2000). Otis and Scott-Dupree (1992) reported on the mortality of colonies infested with tracheal mites, *Acarapis woodi,* in New York state collected data over two winters (1987-1988 and 1988-1989). They found that colony mortality was related to mite infestation levels; colonies with heavy mite infestation levels (82.9%-86.6% infestation of ~100 bee samples) had significantly greater mortality. Furthermore, brood areas in surviving colonies in the spring were shown to be negatively correlated with mite prevalence. In the 1992 season, beekeepers in Sacramento California reported colony losses greater than 50% due to varroa infestations (Kraus and Page 1995). Finley et al. (1996) reported colony losses of 25% to as high as 80% for beekeepers in the northeastern U.S. during the 1995-1996 season. A survey conducted in Indiana attributed a 57% loss of bee colonies during the winter of 1996 due to varroa mites (Hunt 1998). Pennsylvania also had high colony losses the winter of 1996 with a 53% colony mortality (Hunt 1998). In a study of feral colonies of heavily infested areas in California, nine out of ten colonies that were infested in the

spring of 1993 did not survive to the 1994 season (Kraus and Page 1995). The feral colonies observed today are most likely derived from swarms emanatory from colonies treated by local beekeepers (Rinderer et. al. 1999).

Varroa mites prefer to parasitize drone pupae because of the lengthened time in the pupa stage compared to workers and queens (14.5 vs. 12 and 7.5 days, respectfully). As a result of varroa's preference for drone pupae, a number of studies have been conducted looking for adverse effects of varroa on drones. Collins and Pettis (2001), for example, looked at the effect of varroa mite infestation on drone production in the colony, as well as sperm production and the sperm viability of infested drones. The results showed no significant difference between the sperm production and viability of normal drones and drones that had been parasitized by varroa mites during pupation. However, they did find that the number of drones that live to age of reproductive maturity was reduced when varroa mites were found in the cell. In a similar study Rinderer et al. (1999) found that varroa infestation affected the survival of drones beginning on the day of emergence. Only 59.7% of drones emerging in varroa-infested colonies survived their first day of adult life, compared to 97.5% in control colonies. Varroa infestation was also found to have minor negative effects on drone weights, mucus gland and seminal vesicle weights, and the number of spermatozoa produced by parasitized drones.

According to a study conducted in 1994, if one of the two mites, *V. destructor* or *A. woodi*, is found in a hive, then there is a 28% chance that the other is present (Shimanuki et al 1994). Not all, but many hives infested with both mites have predictable symptoms that Shimanuki et al. (1994) called the "Parasitic Mite Syndrome (PMS)." The symptoms included: reduction in the adult bee population, queen supersedure, a spotty brood pattern, crawling adult bees, abandoning the hive, and symptoms that resemble diseases such as American Foulbrood, sacbrood, and European foulbrood. Ball (1985) suggested that varroa mites transmit viruses which impact colonies to a much greater extent than the feeding activities of the mites. In further studies Ball (1989) showed that varroa mites collected from naturally infested colonies transmitted acute paralysis virus (APV), sacbrood, black queen-cell virus, Kashmir bee virus (KBV), and deformed wing virus (DWV) to healthy pupae. In a study looking at the transmission of Kashmir bee virus by varroa mites Chen et al. (2004a) found mites tested positive for the virus,

transmitted the virus to honey bee pupae 70% of the time. They also found a 51% transmission rate from infected mites to virus-free mites when both mites were found sharing the same cell. DWV has been detected in all life stages of the honey bee including adult honey bees with no symptoms of the disease (Chen et al. 2004b). Chen et al. (2004b) suggested that DWV is spread though colonies other ways then direct feeding of mites on adults and pupae based on the detection of the virus in eggs and larval stages of *Apis mellifera*. One potential pathway they suggested is transovarial transmission by nurse bees through feeding the larvae. Research conducted by Hung et al. (1995) served the purpose of determining the role that varroa and viruses play in PMS. They concluded that APV and deformed wing virus are latent virus infections that could be activated perhaps buy stress on the colony, the simple piercing mechanism of the mite when feeding, or foreign substances injected into the pupae by mites during feeding.

Colonies of honey bees infested with varroa or tracheal mites require acaricidal treatment to control infestations that affect colony growth, honey production, and survival (Westcott and Winston 1999). Although there are non-chemical methods such as drone brood trapping and screen bottom boards that help control mite populations, the easiest and most efficient method is to resort to chemical treatments (Lodesani 2004). Over the last 15 years effective varroa mite control strategies in the U.S. have centered on the use of conventional acaricides (Stanghellini and Raybold 2004).

2.2 Miticide Use and The Effects on Honey Bees

The most common means of controlling varroa in the United States has been though the use of synthetic acaricides applied in formulated plastic strips to infested colonies. Up until 1998 the only registered compound for control in the United States was the pyrethroid fluvalinate, sold under the trade name Apistan® (Elzen and Westervelt 2002). Chemical control recommendations are being changed frequently as new products are developed and problems with older treatments become evident (De Jong 1997). Problems were detected with fluvalinate when significant resistance of varroa to the chemical was detected in surveys throughout the U.S. in 1998 (Elzen et al. 1999), and residues of the chemical were found to accumulate in wax (Wallner 1995). Baxter et al. (1998) was the first to document mites that apparently showed resistance to Apistan® in

the United States. This resistance was first noticed in South Dakota within commercially managed colonies. The colonies in South Dakota in which the resistant mites were found originated in Florida (Elzen et al. 1999). Florida beekeepers had been using a steady treatment regime of fluvalinate which led to mite resistance (Eischen 1998). Surveys were conducted in the Western U.S. to check for varroa mite resistance similar to what was detected in Florida and other southern states. Results from 18 states west of the Mississippi showed that fluvalinate was still highly effective against varroa finding little mite resistance (Eischen 1998). With fluvalinate resistance occurring in some states, the beekeeping industry turned to other products for mite control. Since 1998, other compounds have been used from synthetic chemicals to natural oils.

Acaricide treatments are placed in colonies thus exposing not only the mites to the compound, but eggs, larvae, and adult honey bees as well. Whether miticide use can cause non-lethal or lethal effects and/or affect colony development and honey production should be a concern for all beekeepers (Westcott and Winston 1999). Several studies have been conducted showing negative effects of several miticides on the health of colonies and individuals of the hive (Cox et al. 1989, Duff and Furgala 1992, Currie 1999, De Guzman et al. 1999, Rinderer et al. 1999, Westcott and Winston 1999, Ellis et al. 2001, Fell and Tignor 2001, Skinner et al. 2001, Haarmann et al. 2002, Pettis et al 2004).

Research conducted by Rinderer et al. (1999) focused on the effects of fluvalinate on drone honey bees. Their results showed that drone survival after the first day of emergence was significantly reduced compared to untreated drones. They also found exposed drones showed a significant decrease in the body and mucus gland weights. Minor negative effects were also found in regards to seminal vesicle weights and spermatozoa production in treated drones. Fell & Tignor (2001) confirmed in a later study that exposure to fluvalinate during development significantly reduces spermatozoa production in drone honey bees.

Based on the negative effects fluvalinate treatments had on drones (Rinderer et al. 1999), De Guzman et al. (1999) examined the effects of formic acid treatment on the development and production of drones. Formic acid in a gel formulation was tested for the control of varroa in drone producing colonies during the queen-breeding season.

Results of their study were discouraging. Colonies treated with formic acid removed most of the drone eggs from combs and delayed much of the colony's drone production. In addition, treated colonies produced less than half as many drones as untreated colonies (means of 1549 vs. 3800 drones, respectfully) and adult drone longevity was reduced. Similarly, Westcott and Winston (1999) discovered that colonies treated with formic acid had reduced brood areas compared to control colonies, which could potentially reduce colony strength. The amount of honey produced by colonies can also be affected by formic acid, depending on the formulation. Skinner et al. (2001) tested two formulations of 65% formic acid: a gel, and the other with formic acid absorbed into a fibreboard pad. Colonies exposed to formic acid in the fibreboard pad stored less honey than both control colonies and colonies treated with the formic acid gel formulation.

In 1998 an organophosphate compound, coumaphos, was registered for varroa control due to the increasing resistance of mites to fluvalinate (Elzen and Westervelt 2002). Although coumaphos has been quite effective in reducing varroa mite infestation levels, it has had a distinct negative effect on the ability of colonies to rear queens. Haarmann et al. (2002) found that developing queens exposed to as little as one coumaphos-impregnated strip, which is half of the recommended dose, for 24 hours or more suffered a high mortality rate. In addition, queens that completed development showed sub-lethal effects from the coumaphos including physical abnormalities such as lowered body and ovary weights, and lowered mean number of sperm contained in the spermatheca after mating. Fell and Tignor (2001) also observed problems with acceptance of grafted queen cells; reporting only 5% acceptance in colonies exposed to coumaphos compared to 95% acceptance in control colonies.

Wallner (1999) showed that coumaphos accumulates in wax and could potentially impact the ability of colonies to rear their own queens. Based on the evidence of coumaphos having adverse effects on the queen rearing process (Fell and Tignor 2001, Haarmann et al. 2002) and the accumulation of coumaphos in wax (Wallner 1999), Pettis et al. (2004) mixed coumaphos at differing concentrations into wax cups used for rearing queens to investigate the potential effects of coumaphos residues on the ability of colonies to rear queens even after the treatment has been removed from the colony. Young larvae were grafted into queen cups and placed in queenless colonies. Their

results showed that queen cups containing 1000 mg/kg coumaphos led to a 100% rejection by the colony, whereas a 50% rejection rate was observed with cups containing 100 mg/kg, which is the coumaphos tolerance level in beeswax set by the U.S. Environmental Protection Agency (EPA). Queens surviving 100 mg/kg exposure of to coumaphos weighed significantly less than control queens. Collins et al. (2004) continued the same study to determine long-term effects of exposure of queens to coumaphos during their development. They observed that the presence of coumaphos in queen cells can reduce the number of queens still functioning in colonies at six months by as much as 75%.

In 2001, beekeepers began noticing higher than normal varroa populations after treatment with coumaphos, which raised suspicions of developing mite resistance to coumaphos (Elzen and Westervelt 2002). Studies conducted by Elzen and Westervelt (2002) tested four Florida mite populations for coumaphos resistance using a mite population from Texas, which had no history of coumaphos use, as a comparison. They found that all four mite populations tested had significantly less mite mortality compared to the Texas population, confirming that the higher varroa populations in Florida were the result of increased mite resistance to coumaphos.

Botanical products such as essential oils and organic acids have been shown to control mites. They are inexpensive to produce (Lindberg et al. 2000), and have low mammalian toxicity (Quarles 1996). The discovery of varroa's resistance to fluvalinate and fluvalinate's accumulation in beeswax led researchers to focus more heavily on active substances found in nature that would be less risk to beekeepers applying the product, with less accumulation in the wax (Imdorf et al. 1995, Imdorf et al. 1999). A number of essential oils have been tested for efficacy against varroa and tracheal mites with thymol and menthol receiving the most attention (Stanghellini and Raybold 2004). Menthol has been widely used in the United States as a control method for tracheal mites and has proved to be toxic to the mite (Imdorf et al. 1999). Thymol and thymol blends have been widely adopted for varroa control in Europe (Imdorf et al. 1999) and have recently been made available for use in the United States. Both menthol and thymol are naturally occurring chemical compounds found in plants of the mint family (Cox et al.

1989). Although these compounds are natural plant products, they too have been observed to have adverse effects on honey bee colonies.

In a study conducted by Cox et al. (1989) colonies treated with menthol weighed less, produced less honey, and had fewer frames of adult bees than control colonies. Duff and Furgala (1992) conducted a similar study and found a significant reduction in the brood areas of colonies treated with menthol, preventing the queen from expanding the brood upward in the colony. Ellis et al. (2001) found similar problems with thymol application in colonies. They observed that the number of brood cells was significantly reduced in colonies treated with thymol compared to colonies that had been treated with fluvalinate or had bottom screens, thus potentially having an impact on colony strength and survival. Floris et al. (2004) also found a significant reduction in brood area of colonies treated with two different thymol formulations. Thymol residue does occur in the wax and honey of treated hives, however, most of residues evaporate (97% for honey and 89% for wax) 2 weeks after treatment (Floris et al. 2004). Long term studies have shown that residues of thymol in honey remain low and at safe levels when used properly (Imdorf et al. 1999).

2.3 Natural Reproduction of Queen Bees

Both queen and worker bees result from fertilized eggs. The quality and quantity of brood food fed to female larvae determines whether a worker or queen is produced (Winston 1991, Laidlaw and Page 1997, Caron 1999). Queen larvae are fed "royal jelly" which is very similar to the brood food that is fed to young worker larvae, but it contains more mandibular gland secretions, and they are fed larger quantities (Winston 1991). Queens can be produced from worker larvae that are 3.5 days old or younger (Harbo 1986). The development of larvae can change depending on what they are fed within the first three days of larval development (Winston 1991). The development of a queen from egg to emergence takes approximately 16 days, (Winston 1991, Laidlaw and Page 1997) which is shorter than both worker and drone development. Development time of an egg from deposition until hatching varies with values from 48 to 144 hours, averaging 72 hours for all three castes (Winston 1991). Queen larvae will spend 5.5 to 6 days in the

larvae stage followed by another 7 days in the pupa stage (Winston 1991, Laidlaw and Page 1997).

The first task of a newly emerged queen is to seek and destroy potential rivals either existing queens or queens in cells awaiting emergence (Laidlaw and Page 1997, Caron 1999). Virgin queens are smaller than mated queens and tend to be more active and flighty. Virgin queens are fed primarily honey until after mating; honey gives virgins strength and energy for mating flights (Laidlaw and Page 1997). Mating of the queen takes place with multiple drones outside of the hive. Three to 5 days after emergence a queen will take orientation flights to become familiar with the location of her colony; mating can sometimes occur on these flights (Caron 1999). Typically, virgins will make one or more mating flights five to fourteen days after emergence from her cell (Roberts 1944, Woyke 1964, Mackensen and Tucker 1970, Harbo 1985, Caron 1999). If the weather is unfavorable for multiple weeks, and a virgin cannot leave the hive to mate, or she is unsuccessful during mating flights, then she may start laying unfertilized eggs. Queens that only lay unfertilized eggs are known as “drone layers” and will not leave the hive again for mating purposes (Laidlaw and Page 1997).

The number of drones with which a queen will mate varies from queen to queen. It is currently estimated that a queen will mate with between 6 and 24 drones (Woyke 1962, Winston 1991, Neumann et al. 1999) mating with 5 to 10 in rapid succession (Koeniger 1990b). The number of mating flights a queen takes also varies. It is thought that a queen will make repeated mating flights due to a lack of sperm received and stored on the first flight (Roberts 1944, Woyke 1964). Through his research Taber (1954) found virgin queens mate on average 6.5 times over multiple mating flights. Virgin queens allowed only one mating flight had significantly lower sperm (3.27 million) contained in the spermatheca than queens that were able to freely mate (5.73 million) signifying multiple mating flights. Results of progeny of queens allowed to mate once clearly indicated that queens mated with more than one drone on a mating flight based on allele frequencies (Taber 1954). Woyke (1964) observed that queens will repeat mating flights up to three times with the average number of stored sperm increasing with each flight. Two mating flights on the same day can occur but is not likely. Only 6.8% of mated queens flew again on the same day to mate further; most of the queens were found to

mate again on succeeding days. If a queen does conduct a second mating flight on the same day, the duration of the second flight is dependent on the time interval between the first and second flight (Woyke 1962). The longer the time interval between the first and second flights, the shorter the second flight, and the less likely the queen was to mate successfully on it.

The duration of mating flights can vary depending on the success of the flight and season of the year. Flights when queens successfully mate last longer than unsuccessful mating flights. Non-successful mating flights average 11 minutes in comparison to successful mating flights that average 14.4 minutes with some flights lasting as long as 31minutes (Roberts 1944). Woyke (1962) observed a slightly higher average of 21 minutes for a successful mating flight with a second mating flight on the same day lasting 20 minutes. A time interval between two successful mating flights is much shorter (47minutes) than the interval between the first successful mating flight and an unsuccessful mating flight (77 minutes) (Woyke 1962). Mating flights tend to be longer in early spring and then shorten in the summer when the temperatures are warmer and drones are more abundant (Roberts 1944, Laidlaw and Page 1997). Roberts (1944) for example, showed that duration of mating flights decreased from 19.3 minutes in April to 11.9 minutes in June.

Successfully mated queens often return from mating flights with the male endophallus (the “mating sign”) from the last drone with which she mated retained in her sting chamber (Winston 1991), although successfully mated queens can return to the hive without the mating sign (Taber 1954). When a queen returns to the hive with a mating sign the workers assist the queen in removing it (Page 1986). When sperm enters a queen, it travels though the vagina, past the valve fold, and into the lateral oviducts (Snodgrass 1956, Dade 1977). Directly after mating, a queen will have an average of 80-90 million spermatozoa in her lateral oviducts (Koeniger 1986) with as many as 200 million possible (Woyke 1989). The sperm is thought to be unequally divided between the two oviducts, but averages about 11.6μL of total semen volume (Mackensen and Tucker 1970). Within 4.5 hours after copulation the sperm enters the spermatheca (Bishop 1920). Only about 6 million of the 200 million sperm possible eventually reach the spermatheca (Koeniger and Koeniger 1991). A single drone produces more sperm than a spermatheca can hold

(Koeniger 1990b). After returning to the hive the spermatheca is filled, taking about 5% of the total sperm contained in the oviducts; less than 1/10 of the sperm from each drone reaches the spermatheca (Koeniger 1990a).

It was once thought that spermatozoa made their way into the spermatheca by their own activity, most likely activated by chemotactic stimulation (Snodgrass 1956, Dade 1977). However, Ruttner (1956) suggested that the movement of the spermatozoa into the spermatheca is a result of active muscular compression of the abdomen in 1-2 minute intervals. The sperm are forced into the anterior end of the vagina and then into the spermathecal duct. The valve fold prevents further movement along the vaginal tract and helps direct sperm into the spermathecal duct. More recently, studies have shown that motility of the sperm and contraction of the longitudinal muscles in the walls of the spermatheca duct cause a vacuum pump effect that results in about 5 million sperm reaching the spermatheca (Page 1986, Koeniger and Ruttner 1989). Studies conducted by Harbo (1979a) and Woyke (1962) have shown means of 5.00 ± 0.26 million and 5.34 ± 0.12 million sperm, respectfully in the spermatheca of queens at onset of oviposition. A higher spermatozoal average of 9.77 ± 0.97 million has also been found in the spermathecae of newly mated queens (Szabo and Heikel 1987). Within 24 hours any sperm that did not pass into the spermatheca is discarded from the oviducts and vagina along with any mucus (Woyke 1964, Snodgrass 1956, Koeniger and Koeniger 1991).

There has been much debate on whether sperm competition exists in honey bee spermatozoa. Because the queen mates multiple times and stores sperm from multiple males, the potential for sperm competition cannot be ignored (Schluns et al. 2004). Sperm competition implies that there are physiological or genetic differences in spermatozoa which affect their ability to fertilize eggs (Harbo 1988). Sperm competition may include competition for leaving the spermatheca, for reaching for the egg, penetrating the egg, or any combination of these activities (Harbo 1990). Incomplete mixing of spermatozoa from different drones has been demonstrated in *Apis mellifera* (Page 1986), with each drone contributing variable amounts of viable sperm (Woyke and Jansinski 1978, Collins 2004). Harbo (1988, 1990) found a unidirectional difference in offspring of queens inseminated with the same mixture of spermatozoa suggesting that non-mixing of spermatozoa is not the cause of unequal distributions from each drone. Harbo (1988,

1990) suggested that a form of competition exists that shifts the advantage to a particular population of sperm at different times, perhaps by environmental factors such as the time of year, when there is a lot of sperm released for fertilization, or when the queen has small amounts of spermatozoa in her spermatheca. On the other hand, the use of phenotypic markers has shown that sperm from all of the mated drones become randomly distributed within the spermatheca (Page et al. 1984). Relatively constant levels of paternal types have been observed in worker offspring, signifying little or no sperm competition (Collins et al. 2006). A study by Schluns et al. (2004) looked at mating order and semen volume in relation to sperm utilization patterns in honey bees. The results showed that the order in which drones mate with a queen has no determination on the frequency of their offspring however, the amount of semen a drone contributes matters. Drones should maximize the number of sperm they produce, but should not apply mating tactics to be first in the mating sequence.

Development of eggs occurs in the ovaries, each composed of up to 180 ovarioles (Woyke 1985). The mature egg passes through the lateral oviduct into the median oviduct and later into the vagina. The insemination of the egg takes place in the vagina as the egg passes the orifice of the spermathecal duct (Snodgrass 1956). The queen is able to release small quantities of semen from the spermatheca to fertilize eggs as they pass the opening of the spermathecal duct (Dade 1977). When a queen fertilizes an egg she uses 1/153,000th of the spermathecal volume to fertilize one egg; thus the number of spermatozoa expelled for fertilization depends on the concentration of the spermatheca (Koeniger 1990a). The volume of sperm released by a queen may not be constant (Harbo 1979a). Harbo (1979a) suggests that the number of spermatozoa released for fertilization decreases over the course of the queens reproductive life. Just below the opening of the median oviduct is a muscular fold in the floor of the vagina that is known as the valve fold. This flap like valve may be used to press eggs against the opening of the spermathecal duct as sperm are released from the spermatheca (Dade 1977).

2.4 Spermatheca

For a queen's entire reproductive life the supply of spermatozoa is limited to those she obtained before she starts laying (Harbo 1979a). The amount of sperm held in

the spermatheca decreases as the queen ages (Szabo and Heikel 1987, Lodesani et al. 2004), for many spermatozoa are released each time an egg is laid (Harbo 1979a). The amount of spermatozoa the in spermathecae of queens 24 months of age is significantly lower than that of queens 2 months old, but not significantly lower when compared to queens aged 12 months (Lodesani et al. 2004). Harbo (1979a) observed queens using half of their spermatozoa within 6 months of egg-laying. In contrast, the viability of sperm held in the spermatheca of queens 24 months of age was significantly lower than queens of 12 and 2 months of age (Lodesani et al. 2004). Lodesani et al. (2004) found that sperm collected directly from drones were less viable (32.8 ± 4.1% dead sperm) than spermatozoa contained in inseminated queens (20.5% dead sperm) analyzed two months after insemination, leading them to speculated that sperm in the spermatheca undergo an initial selection for quality.

The spermatheca is the primary sperm storage organ in queen honeybees. It is a small spherical gland that lies over the vagina and is connected to it by a short duct. At the top of the spermathecal duct is a branched gland (spermathecal gland) that connects to and hugs the surface of the spermatheca (Snodgrass 1956, Verma 1974, Dade 1977). The two-branched spermathecal gland plays a role in the delivery of spermatozoa into the spermatheca. When a single branch of the gland is removed, there is no effect on the amount of spermatozoa contained in the spermatheca, but after the removal of both branches only 1-2% of the normal amount of spermatozoa entered the spermatheca (Koeniger 1969).

The spermatheca has a diameter ranging from 0.9-1.30mm, a volume of 0.45-1.15μL (Verma 1974), and it is surrounded by a dense tracheal network referred to as the "tracheal net." The tracheae serve as a means to deliver oxygen to tissues. Thus, it has been assumed that the spermatozoa require a high oxygen supply based on the presence and complexity of the tracheal net (Poole 1972). However, this assumption has been debated. Koeniger (1969) removed the trachea surrounding the spermatheca and found that mated queens became drone layers, indicating that the spermatozoa were rendered non-functional or dead. Poole and Taber (1970) on the other hand, questioned the importance of the trachea with regard to the delivery of oxygen to the spermatozoa. The authors concluded the respiratory supply to the spermatozoa is of secondary importance

and that the primary function is to supply oxygen to the epithelial cells of the spermathecal wall. Poole (1972) also refuted Koeniger's assumption because of the lack of evidence of direct respiratory connections between the spemathecal lumen and the tracheal network outside the wall. Poole (1972) found that the epithelial cells of the spermatheca die promptly after binding or removing even a small part of the tracheal net, indicating that the spermathecal wall is dependent on some function of the tracheal network.

Sperm can be stored for many years in the spermatheca of a queen until the queen dies (Snodgrass 1956, Schluns et al. 2004). Much debate has taken place as to the motility of sperm stored in the spermathecae of queens. The current understanding is that spermatozoa are stored in an immobile state but motility can be restored by spermathecal gland secretions (Flanders 1939, Lensky and Schindler 1967). The lack of motility of spermatozoa in the spermatheca also suggests that their metabolic activity is very low. The low metabolic rate of immobile sperm has been confirmed by Verma (1974) in studies on the *in vitro* storage of honey bee sperm.

Very little is known about the biochemical composition of the spermatheca. The pH of the spermatheca is 9, with the spermathecal gland at a pH of 7 (Lensky and Schindler 1967). The spermatheca of both mated and virgin queens contains glucose, trehalose and fructose (Alumot et al. 1969). The high concentration of trehalose in the spermatheca suggests that the spermathecal gland converts glucose to trehalose to be used later as a source of energy by stored spermatozoa (Verma 1974). Verma (1973) observed high concentrations of Na^+ and K^+ in the spermatheca of queens. He suggested that the high concentrations of these cations may induce inactivity of the stored spermatozoa. Gessner and Gessner (1976) found the concentration of K+ to be 8 times higher in the spermathecal fluid than in the heamolymph of the queen honey bee. They also found that the spermatheca epithelium generated an electrical potential difference of 21 mV, lumen positive suggesting that the secretion of K+ is an active energy dependent process.

2.5 Natural Drone Reproduction

Drone honey bees develop from unfertilized eggs laid by queens or workers. The development of a drone takes 24 days which is longer than both worker and queen

development (Caron 1999). Drone populations fluctuate with the season, reaching their highest peaks in spring, declining in the summer, and reaching their lowest point in the fall (Peng et al. 1992). Drones are driven out of colonies in the fall or when resources are scarce (Winston 1991, Caron 1999). During their development drones require proper food (pollen and honey) in order to develop and mature the reproductive cells and organs necessary for mating (Laidlaw and Page 1997). Sexual maturation time in drones varies from as early as 6 days (Harbo 1986) to as late as 12 days (Snodgrass 1956).

Once drones are sexually mature they will leave the hive to go on mating flights. Drones have been observed leaving the hive as early as 12:30 pm (Roberts 1944) and returning as late as 6:00pm (Cramp 1998). In 1923 it was first recognized that drones gather in specific aerial locations for mating (Buteel-Reepen 1923). The aerial locations where drones gather are known as Drone Congregation Areas (DCA's) and are defined as definite locations where drones regularly assemble, independently of the presence of a queen (Jean-Prost 1986, Loper et al. 1987). Congregation areas are found at least 90-120m from an apiary with areas further away attracting higher numbers of drones (Winston 1991). Drones easily locate DCA's and appear within DCA's on their first mating flight, possibly locating these areas where specific physical land features are found (Loper et al. 1987, Winston 1991). A single drone can visit more than one DCA during his lifetime with the potential to visit two or three different DCA's in a single afternoon (Ruttner and Ruttner 1966). The location of congregation areas can remain constant over several years (Loper et al. 1987, Winston 1991, Cramp 1998, Caron 1999), with a few remaining constant for 15 years (Koeniger 1986). The shape of the congregation area, described as a tilted column, and the distribution on drones within it is constantly changing (Loper et al. 1987).

The number of drones present at a congregation area varies. Gary (1963) for example found that drone swarms ranged from 100-300 drones when 1-5 queens were displayed simultaneously. Koeniger (1990b) has observed a single queen encountering more than 1000 drones at DCA's. The number of drones attending a DCA depends on many factors including the time of day, weather conditions, and the attractiveness of an area (Winston 1991). When a queen flies into a drone congregation area, many drones will pursue her in a comet-like group attracted by the queen mandibular pheromone, (E)-

9-oxo-2decenoic acid (9ODA) (Jean-Prost 1986, Winston 1991), and by visual cues (Gary 1963, Strang 1970). Successful mating with a queen occurs for less that 1% of drones, and results in death (Winston 1991, Caron 1999). DCA's reduce survival risks and energy expenditure of the queen because queens do not have to "find" each mate (Koeniger 1990a). Also, fitness benefits accrue to the queen by traveling to DCA's from reduced chances of inbreeding (Koeniger 1986).

The reproductive organs of the male honey bee include a pair of testes, their outlet ducts which enlarge into seminal vesicles, a pair of large mucus glands, a single ejaculatory duct, and a penis (Snodgrass 1956). Spermatozoa are produced in the two testes during the pupa stage of development (Woyke 1985). Spermatozoa begin to move from the testes to the seminal vesicles when the drone is about 3 days of age (Harbo 1986, Snodgrass 1956, Woyke 1985). Spermatozoa remain in the seminal vesicles until the act of mating. Drones emerge from their cells as adults possessing the number of sperm that they will have for the remainder of their life, unlike mammals, which continue to produce sperm post puberty.

Each drone produces between 1.50 and 1.75 μL semen containing about 11 million spermatozoa (Woyke 1962). The amount of sperm a drone produces is dependent on its size and origin (egg from a queen vs. laying worker). Schluns et al. (2003) compared average spermatozoa numbers of drones from the same colony reared in either worker cells or drone cells. Worker bees are reared in smaller cells (5.2-5.8 mm diameter; Dietz 1992), whereas significantly larger cells (6.2 mm diameter) are used for rearing drones. The differences in body size among drones are mainly determined by this variation in brood cell size (Schluns et al. 2003). The results showed that smaller drones reared in worker cells produce significantly fewer spermatozoa (7.5×10^6) than normal sized drones reared in drone comb (11.9×10^6 spermatozoa). Gencer and Firatli (2005) found that when queenright and laying worker colonies were given drone combs for high drone production, the weight of drones produced in queenright colonies were 17% heavier than those produced in laying worker colonies. The mean number of spermatozoa of drones from queenright colonies (12.01×10^6) was also significantly greater than drones from laying worker colonies (10.17×10^6).

Spermatozoa that drones produce are very long and thread-like. The spermatozoa of the honey bee resemble sperm of other insects, but are much longer than those of mammalian species (Verma 1974). Spermatozoa are elongate thin cells, approximately 250μm long and 0.7 μm wide (Short and Goncalves 1986, Dade 1977, Peng et al. 1993). The head of a honey bee spermatozoon is asymmetric and comprised of the acrosomal complex tip and the oval-shaped nucleus (Lensky et al. 1979, Woyke 1984, Peng et al. 1993). The head measures 12μm in length (Peng et al. 1993) and contains the nucleus that holds the genetic material transmitted by the drone (Dade 1977). The tail consists of an axoneme or flagellum, two adjacent mitochondrial derivatives of unequal size (Lensky et al. 1979), and two triangular shaped accessory bodies (Woyke 1984, Peng et al. 1993). With the use of a light microscope, there is no distinct line of differentiation between the head and the tail (Short and Goncalves 1986, Locke et al. 1990). The lack of demarcation between the head and the tail accompanied by its extreme length make bee spermatozoa difficult to count without the aid of nucleic stains.

The semen of drone honey bees is a cream colored fluid containing two main elements: sperm and seminal plasma. The pH of ejaculated sperm ranges from 6.0 to 7.1 and contains the sugars: fructose, glucose, and trehalose (Blum et al. 1962, Verma 1974). These sugars appear in the seminal plasma and are most likely the source of energy for the spermatozoa (Short and Goncalves 1986).

2.6 Natural Mating

Natural mating occurs far away from the queen's hive to ensure the queen mates with several unrelated drones (Koeniger 1990a). Natural mating of honeybees is hard to observe because it takes place during flight (Gary 1963, Koeniger and Koeniger 1991). To enable mating observations Gary (1963) designed a tethering device to hold virgin queens at low altitudes. Later studies (Gary 1969, Koeniger 1981, Koeniger 1988) were conducted using the same tethering techniques and were documented on film, increasing the understanding of honeybee mating behavior. Gary's (1963) work on mating behavior settled a debate on the mating position of the queen and drone during copulation. Gary (1963) observed drones in the dominant position on top of the queen opposed to other

speculations of a “face-to face” position (Shuck 1882, Bishop 1920) or a queen dominant position (Rothschild 1955).

The mating behavior of *Apis mellifera* has been described by Koeniger (1986) and Koeniger and Koeniger (1991) and is summarized below:

> Flying drones mount queens from behind and make contact. The drone grasps the queen’s abdomen with his legs and waits for the queen to open her sting chamber. The drone inserts his endophallus into the sting chamber, stops moving, and his body swings back. The drone is anchored to the queen by mucus in the endophallus filling the sting chamber and sticky cornua secretions increase the strength of the attachment. The queen then contracts her bursa copulatrix (vaginal orifice) squeezing the endophallus which results in sperm transfer to the median oviduct. After full eversion of the endophallus, the drone separates from the queen and falls to the ground leaving mucus and the corneal secretions as a “mating sign” in the queen’s sting chamber.

Termination of mating is audible, recognized by a snapping sound caused by compressed air in the drone genitals (Gary 1963, Winston 1991). The copulation period takes place in under 5 seconds (Koeniger and Koeniger 1991).

The role of the “mating sign” has been speculated as a mating plug which prevents or complicates future mating of the queen (Thornhill and Alcock 1983, Koeniger 1990a). This role was hypothesized based on the sticky cornua secretions which fill the queen’s sting chamber. However, genetic experiments and sperm measurements in the oviducts later concluded that multiple mating occur during a single mating flight (Koeniger 1990a). Subsequent drones were observed inserting their endophallus ventral of the mating sign and removing it with a special patch of hairs on the endophallus (Koeniger 1984, 1986, 1990a). The drone then mates and leaves his own mating sign. The mating sign keeps the sting chamber of the queen open and may protect the endophallus of the subsequent drone from the stinger of the queen (Koeniger 1986).

The mating sign is white, partially covered by a thin orange layer, and is clearly visible and contrasts in color with the queen (Koeniger 1990a, 1990b). Koeniger (1990a, 1990b) found that drones significantly prefer to copulate with queen models with a mating sign. Further, in other studies they observed drones copulating significantly more often with queen models which were a different color then themselves. The drone leaving his mating sign in the queen seems to facilitate further mating, functioning as an optical marker. Both the preference of the mating and sign and the discrimination between colors of queens shows that size, form, and colors are used for visual orientation in mating.

2.7 Sperm Collection and Storage

The collection and storage of honey bee spermatozoa is commonly practiced for use in instrumental insemination. Drones are not always available especially in places with cold winters. The potential to store sperm allows breeders the freedom to mate queens after drone production has ended (Short and Goncalves 1986). A practical means to store honey bee semen would enhance the ability to select and maintain superior honey bee stocks (Collins 2000a), particularly for the selection of honey bees that show resistance to varroa, tracheal mites, and American Foul Brood (AFB).

Early work on semen collection involved the removal of the seminal vesicles though dissection. Semen was taken up into a syringe from an opening made in one end of the seminal vesicle (Mackensen 1955). The current method of honey bee sperm collection involves taking sperm into a syringe from the completely averted endophallus. The process of partial eversion involves crushing the thorax between the thumb and forefinger. During partial eversion, the sperm and mucus are ejaculated into the penis bulb. Complete eversion occurs with increasing pressure of the abdomen until semen appears along with mucus on the tip of the penis (Mackensen 1955, Collins 2004).

Taber and Blum (1960) were responsible for some of the early work on the effects of different diluents and temperature on the viability of sperm stored in capillary tubes with heat sealed ends. Semen was successfully stored at room temperature for 4 weeks; however, problems arose from the contamination of sperm with microorganisms. Poole and Taber (1969) improved sterilization techniques and added different antibiotics during the collection of the sperm to reduce bacterial growth. As a result of their research, semen

could be successfully stored at room temperature for 3-4 months. Streptomycin is still used in buffer solutions for the collection of semen. Harbo (1974) improved the process by modifying a capillary tube to serve as both an insemination tip and a storage container. Prior to his work, semen was collected with a syringe and then transferred into a storage tube and then back into the syringe for insemination. Harbo's design provided a means to make short-term (1-10 weeks) sperm storage more practical.

A study conducted by Harbo and Williams (1987) found that temperature at which sperm is stored has an effect on the amount of sperm taken into the spermatheca during insemination. They found that sperm stored between 20º and 25ºC (room temperature) resulted in the highest sperm numbers contained in the spermatheca. Collins (2000b) has done more recent work on the survival of honey bee sperm stored at above-freezing temperatures. She observed survival of 70-80% of spermatozoa stored at room temperature up to 6 weeks after collection, with no significant decrease in viability from the initial collection. However, after 6 weeks of storage, sperm viability significantly decreased from 80% to 58% at week 9. An earlier study by Taber et al. (1979) found that the sperm viability of sperm stored at room temperature can be restored to normal values by mixing the aged sperm with fresh sperm before insemination. They speculate that the reversal of senescence of aged sperm in the presence of fresh semen may be accomplished by a chemical transfer of energy.

In a subsequent study, Collins and Pettis (2001) observed considerable variation in the viability of sperm samples (83-100%) collected from individual drones. Extremely low viability of 20.8% has also been found in pooled samples collected from many drones (Collins 2000a). Collins (2004) addressed two major factors that may contribute to this variation: temperature of the semen, and physical damage that may occur from handling. The manner in which semen is collected was found to be a more significant factor than the temperature of the buffer used at the time of sperm collection. Collins found that the technique yielding the highest viability involved collection of spermatozoa directly from the seminal vesicle followed by washing semen from the endophallus of an ejaculated drone. The standard practice of collecting semen using a syringe was the least effective. The recommended technique for the collection of sperm with the highest viability is to utilize a washing method, given that obtaining semen from the seminal

vesicles is impractical for insemination purposes. Kaftanoglu and Peng (1980) were the first to develop a washing technique for collecting honeybee semen. No significant differences were found in the amount of brood produced by queens inseminated with semen collected from the washing technique compared to traditional syringe collection, and naturally mated queens, validating its usefulness in insemination procedures.

Both the age of the drone used for collection, and the time of year in which the semen is collected may have an effect on the quantity and quality of the semen collected. Woyke and Jasinski (1978) found that drone age at the time of semen collection plays a role in insemination success. They observed lower numbers of sperm entering the spermatheca of artificially inseminated queens as the age of drones increased. Locke and Peng (1993) took at closer look at the effects of drone age on semen quality though the use of viability assays *in vitro,* supporting Woyke and Jasinski's conclusions. They observed significantly lower sperm viability in drones 4 and 6 weeks old compared to fully matured 2-week-old drones. Locke and Peng (1993) speculate that the decline in sperm viability may be an indicator of a natural spermatozoan aging process, since spermatogenesis occurs only during the pupa stage in honey bees, unlike other insects in which spermatogenesis continues throughout adult life (Bishop 1920). A study conducted by Kumar and Kaur (2003) in India suggests that semen used for artificial insemination should be collected in the spring. The results of their study showed higher spermatozoal production of drones in the spring compared to the fall. Also higher carbohydrate and proteins were found in semen when collected in the spring. High carbohydrate and protein counts meet the energy demands of spermatozoa for better sperm motility and nutrition during storage (Kumar and Kaur 2003).

Many studies (Sawada and Chang 1964, Harbo 1977, 1979b, 1983, Kaftanoglu and Peng 1984, Short and Gonclaves 1986, Peng et al. 1992) have been conducted on long term (1-2 year) storage of honey bee sperm through preservation in liquid nitrogen. Honey bee sperm cannot survive temperatures of -196°C when packaged in a capillary tube and placed in liquid nitrogen; a compound must be added to act as a cryoprotectant (Short and Goncalves 1986). Kaftanoglu and Peng (1984) examined different diluents, cryoprotectant concentrations, and rates of freezing and thawing sperm samples.

The objective of low temperature sperm storage is to have sperm available for insemination at any time of the year. The goal is for queens inseminated with sperm stored at low-temperatures (-196°C) to perform just as well as queens inseminated with fresh semen. The quality of stored semen has been tested by measuring the amount of fertilized worker brood produced by queen bees inseminated with cryopreserved semen (Peng et al. 1992). Equality of queens inseminated with fresh semen and semen preserved in liquid nitrogen has not been achieved. Kaftanoglu and Peng (1984) found that brood production by queens inseminated with low-temperature stored semen is not sufficient to maintain a colony. Studies conducted by Peng et al. (1992) investigated the effect of rapid freezing and thawing on sperm fertility, explaining observations seen by Kaftanoglu and Peng (1984). Peng et al. (1992) observed damage to the plasma membrane, destruction of the sperm acrosome, and splitting of mitochondrial derivatives from the flagellar axoneme, negatively affecting the viability of the sperm.

2.8 Viability Assessment of Honey Bee Sperm

Techniques for accurately measuring sperm viability are essential in evaluating sperm quality (Locke et al. 1990). The success of artificial insemination is highly dependent on the quality of sperm (Peng et al. 1990). Early work conducted in sperm viability assessment involved looking at the quality of brood produced by inseminated queens (Peng et al. 1990, Collins and Donoghue 1999). Two more recent methods to measure viability are absence of mobility and stain exclusion by live cell membranes (Locke et al. 1990). Supravital staining techniques are often used in assessing the effects on mammalian sperm before freezing and after thawing (Peng et al. 1990). Assays for semen quality are important tools for assessing male fertilizing potential in all artificial insemination programs (Saacke 1984).

Locke et al. (1990) experimented with using two fluorescent stains, propidium iodide and Hoechst 33342 (H342), for a new supravital staining procedure for honey bee spermatozoa. Both stains selectively bind to DNA in the nucleus of sperm cells. H342 penetrates cell membranes causing the nucleus of all the sperm cells to fluoresce a bright blue color (Visser 1981). Propidium iodide (PI) penetrates only damaged membranes, causing the nucleus of the damaged sperm cells to fluoresce bright red (Krishhan 1975).

The percentage of drone sperm non-viability is estimated by the sperm stained by PI. Based on the results of Locke et al (1990) studies, they recommended that a 1μL semen sample be incubated in a 100-150μL solution containing 5μg/ml H342 and 10μg/ml propidium iodide in a modified Kiev solution. The samples should then be incubated for 15-20 minutes, mounted on a slide, and examined using fluorescence microscopy.

In contrast to the viability assessment of free swimming sperm conducted by Locke et al. (1990), the goal of Peng et al. (1990) was to assess honey bee sperm viability as a dry smear for permanent preservation of samples. Seven different staining techniques were modified and tested for differential staining of live and dead sperm. Peng et al. (1990) found that an eosin Y staining method was found to be a simple technique for determining honey bee sperm viability. The eosin Y stain was mixed with an equal volume of diluted sperm and allowed to incubate for 1-2 minutes. A smear was taken of the sample and evaluated with a phase microscope. The live cells were clearly stained bluish purple whereas the dead cells were stained bright yellow.

After validation of fluorescent stains SYBR-14 and Calcein-AM use in conjunction with propidium iodide in sperm viability assays in mammalian and avian species, Collins and Donoghue (1999) conducted a study using those same stains with honey bee sperm. They tested both SYBR-14 and Calcein-AM separately with PI with methods similar to those used by Locke et al. (1990). Both SYBR-14 and Calcein-AM stain living cells green, while PI stains dead cells red. Their results found that both living stains fluoresced honey bee spermatozoa, but the SYBR-14 stain provide better distinction between the living and dead cells.

3. The Effects of Fluvalinate, Coumaphos, and Thymol on the Production and Viability of Drone Honey Bee (*Apis mellifera* L.) Spermatozoa

3.1. Introduction

Since the introduction of *Varroa destructor* (Anderson and Trueman) in the United States in 1987 effective control strategies have centered on the use of conventional acaricides (Stanghellini and Raybold 2004). Acaricide treatments placed in colonies expose not only the mites to the chemical, but eggs, larvae, and adult honey bees as well. Whether miticide use can cause non-lethal or lethal effects and affect colony development and honey production is a concern for all beekeepers (Westcott and Winston 1999).

Previous research has alluded to potential effects of miticides on the survival and reproductive physiology of drones. In a study on the effects of fluvalinate (Apistan®) on drones, Rinderer et al.(1999) found a significant decrease in the body and mucus gland weights of exposed drones. Drone survival after the first day of emergence was also significantly reduced compared with drones from control colonies. Negative trends were seen with Apistan® treatments with regard to seminal vesicle weights and spermatozoa production in treated drones, but significant differences were not observed. In a similar study, Fell and Tignor (2001) found that exposure to fluvalinate during development significantly reduced spermatozoa production in drone honey bees. Formic acid, another compound used in mite control, has been shown to decrease drone production and adult survival in treated colonies (De Guzman et al. 1999).

Coumaphos, an organophosphate compound, has been shown to have a distinct negative effect on the ability of colonies to rear queens. Several reports (Collins et al. 2004, Pettis et al. 2004, Haarmann et al. 2002, Fell and Tignor 2001) provide findings of negative effects of coumaphos on queens, including high mortality, lowered body and ovary weights, decreased mean sperm numbers held in the spermatheca, lowered acceptance of larvae grafted for queen cells, and reduced numbers of queens functioning in colonies by 75% after 6 months. Coumaphos and the essential oil miticides, thymol and menthol, have been studied for their effects on colony and queen performance, but the effects of these compounds on drones is unknown.

The effects on the quality and quantity of drones for mating should be a concern for all beekeepers. Queens mating with insufficient drones may result in an early supersedure of queens (Camargo and Gonclaves 1971). It is critical to determine if drones from fluvalinate, coumaphos, and thymol treated colonies that survive to mating age are capable of providing sufficient and healthy spermatozoa for successful matings.

The objectives of this study were to treat colonies with the recommended labeled concentrations of fluvalinate (Apistan®), coumaphos (Check Mite+), and Apilife VAR® (74.1% thymol, 16.0% eucalyptus oil, 3.7% L-menthol, and 6.2% other) to investigate the effects of these miticide treatments on sperm production stored and viability in *Apis mellifera* drones. This study was undertaken to determine whether sub-lethal effects on drone reproductive physiology could contribute to the reoccurring problems in queen and colony health and performance such as queen failure and supersedure.

3.2. Materials and Methods

3.2.1. Drone Rearing

Drones were produced in standard 10 frame colonies with new un-treated combs. Naturally mated sister queens grafted from the same mother were placed in each standard full depth hive. Each colony was given a frame of drone comb that had been recently drawn out to minimize the possibility of miticide residues. A total of 16 colonies were set up for the experiment, but only 8 colonies were used, 2 colonies per treatment and control. Apistan® and Checkmite+ (10% fluvalinate or coumaphos in plastic strips) were administered to the colonies using two strips, one placed next to the drone comb. Plastic strips were left in the colony for 6 weeks. Apilife VAR®, formulated in floral foam tablets, were broken into four pieces, enclosed in #8 mesh wire screen to prevent bee chewing and removal, and placed on the top corners of the hive body. Apilife wafers were replaced with fresh wafers every 10 days.

Queens were confined to a single frame of drone comb using a divider made from a plastic queen excluder. Within 1-2 days after queen confinement, drone cells were checked for sufficient numbers of eggs and the dividers were removed. Two days before drones emerged (24 days after egg laying) the drone frame was raised to a full depth hive body above a queen excluder. When drones emerged, they were marked on the thorax

with enamel model paint and placed back into the hive. Marked drones remained in the colony 14- 20 days to allow for full sexual maturation. Drones to be sampled for viability and sperm counts were collected in afternoons at the colony entrance by hand or using an entrance drone trap. Drones that were not used immediately were held overnight in cages made with queen excluder material, allowing workers to enter and feed the drones (Collins 2004a). A group of 16 drones per treatment was collected and used for either viability or sperm number assessment.

3.2.2. Colony Mite Levels

At the end of the summer season varroa mite levels were estimated in treatment and control colonies using a sticky board method. A 12 x 12 inch fly trap paper was covered with black plastic mesh and placed on the bottom boards in each hive. Sticky boards were removed 24 hours after placement in the colonies and total mite counts were recorded for each hive. A total of 13 hives were tested for mite levels; four control colonies and three colonies for each treatment. An average mite drop for each treatment and control was calculated.

3.2.3. Evaluation of Sperm Viability

Prior to sperm sampling, drones were placed in a flight cage to warm up, increasing the success of ejaculation during stimulation (Collins 2004a). Mature drones were stimulated to ejaculate by crushing the thorax. Full eversion of the endophallus for semen collection was accomplished by applying pressure to the abdomen (Harbo 1985, Moritz 1989). Approximately 1μL of semen was collected from each drone using a washing method similar to Kaftanoglu and Peng (1980). The tip of the genitalia was rinsed with 300μL of modified Kiev buffer (Kaftanoglu and Peng 1984) into a 1000μL centrifuge tube. Modified Kiev solution (MKS) was used to dilute the semen due to its similar osmotic pressure and pH as the semen ejaculate (Lensky and Schinder 1967, Verma 1973). The MKS also contained 0.05% streptomycin sulfate and was filtered (0.6 μm pore size) before use (Locke et al. 1990).

Sperm viability was assessed using fluorescent stains (Locke et al. 1990). Stain stock solutions were composed of 0.9% NaCl and either 500 μg/ml propidium iodide (Sigma Chemicals, St Louis, Mo.) or 1000 μg/ml Hoechst No. 33342 (H342) (Sigma Chemicals, St Louis, Mo.)(Locke et al.1990). The diluted sperm solution (300μL) was

combined with 100μL of a working stain solution (950μL MKS, 10μL H342 solution, and 40μL propidium iodide solution), and was incubated at room temperature for 15-20 minutes. Sperm samples were counted directly after the 15-20 minute incubation time to obtain the highest possible sperm viability, and to reduce inconsistencies between samples. Propidium iodide enters dead cells staining them red, while H342 adheres to nucleic acids in live cells and fluoresces green. A small 15 μL aliquot of the sample was placed on a glass slide with a cover slip, and observed under 40X magnification on a Nikon Eclipse E600 Epi-fluorescent microscope equipped with a BV-2A filter. Each sample, which represented one drone, was counted 4 times using 200 sperm per count. A total of 16 drones were sampled for each treatment, 8 from each colony, totaling 64 drones. In each count the sperm was scored live or dead based on the color of fluorescence. An average percentage of live sperm from the four counts was used for statistical analysis.

3.2.4. Evaluation of Sperm Numbers

Eight drones collected from each colony were dissected and one seminal vesicle was removed from each drone. A total of 16 seminal vesicles for each treatment were analyzed, as two colonies were used for each treatment. Each seminal vesicle was placed in a 1500μL centrifuge tube and was gently macerated using a small plastic pestle. While macerating, an effort was made to insure that the seminal vesicle remained in contact with the tube and pestle, so that the sperm were thoroughly dispersed in the diluent. The pestle was then rinsed over the centrifuge tube with 100μL MKS four times to insure the removal of any adhering sperm from the pestle. After rinsing, the centrifuge tube contained a 400μL diluted sperm sample. Estimates of total sperm number for each seminal vesicle were made using a Bright-line Hausser Scientific hemacytometer and an Olympus BX40 light microscope equipped with phase-optics. Four aliquots of diluted sperm were counted for each sample. An average count of sperm was determined for each drone and a final concentration of number of sperm per seminal vesicle was calculated using the formula:

Number of sperm per seminal vesicle = average sperm count per square * 25 * 10 * dilution factor

3.2.5. Statistical Analysis

Experiments were set up as a complete randomized design. All statistical analyses were computed using the SAS 9.1 software package. Prior to analysis a Shapiro-Wilk test was performed to check for deviation from normality of the samples. The data were analyzed using a one-way analysis of variance (ANOVA). A Fisher's LSD test was performed to make pairwise comparisons among treatment means. An arcsine transformation was used on all sperm viability data to meet the assumptions of the analysis of variance model.

3.3. Results

Sperm Viability

Mean (± SE) percent live spermatozoa in *Apis mellifera* drones are shown in Figure 3.1. The viability of sperm from all 64 drones examined varied from 88.1% to 99.9%. Mean (± SE) sperm viability of sperm varied from a low of 93.2% ± 0.7 in drones exposed to coumaphos, to a high of 98.5% ± 0.4 for drones reared in control conditions. Exposure of drones to miticides during development was a significant factor in sperm viability (df = 3, 60; F = 30.17; $P < 0.0001$). Sperm viability in drones exposed to coumaphos during development was significantly lower than the sperm viability of control drones (Figure 3.1). Rearing drones in colonies treated with Apilife VAR® or fluvalinate had no significant affect on sperm viability.

Numbers of Spermatozoa

The values for mean sperm content in drones are shown in Figure 3.2. Sperm numbers ranged from 1.80 x 10^6 to 8.07 x 10^6 spermatozoa per seminal vesicle. Mean (± SE) sperm number of 5.99± 0.28 x 10^6 was found for the control drones and the treatment means for fluvalinate, thymol, and coumaphos were 5.33± 0.21 (S.E.) x 10^6, 4.78± 0.36 x 10^6, and 2.98± 0.24 x 10^6 spermatozoa, respectfully. The Shapiro-Wilk test did not reveal any significant departure from the normal distribution (W = 0.984169; P< 0.5843). The one-way ANOVA yielded a highly significant difference between sperm numbers in control drones and drones exposed to thymol or coumaphos (df = 3, 60; F = 21.49, P< 0.0001). Drones exposed to coumaphos during their development had significantly lower sperm counts than drones of control, fluvalinate, and thymol treatments (Figure 3.2).

Varroa Mite Loads

Mite counts in colonies ranged from 5 to 56 mites per colony. Average mite counts per treatment are shown in Table 3.1. Control colonies had the highest mite drop in 24 hours averaging 46.0 ± 4.7 (S.E.) mites, which was significantly higher ($P < 0.0009$) than the other three treatments. Colonies treated with fluvalinate, coumaphos, and Apilife VAR® averaged 11.0 ± 4.6 (S.E), 15.3 ± 6.4 (S.E.), and 12.0 ± 1.7 (S.E.) mites, respectfully.

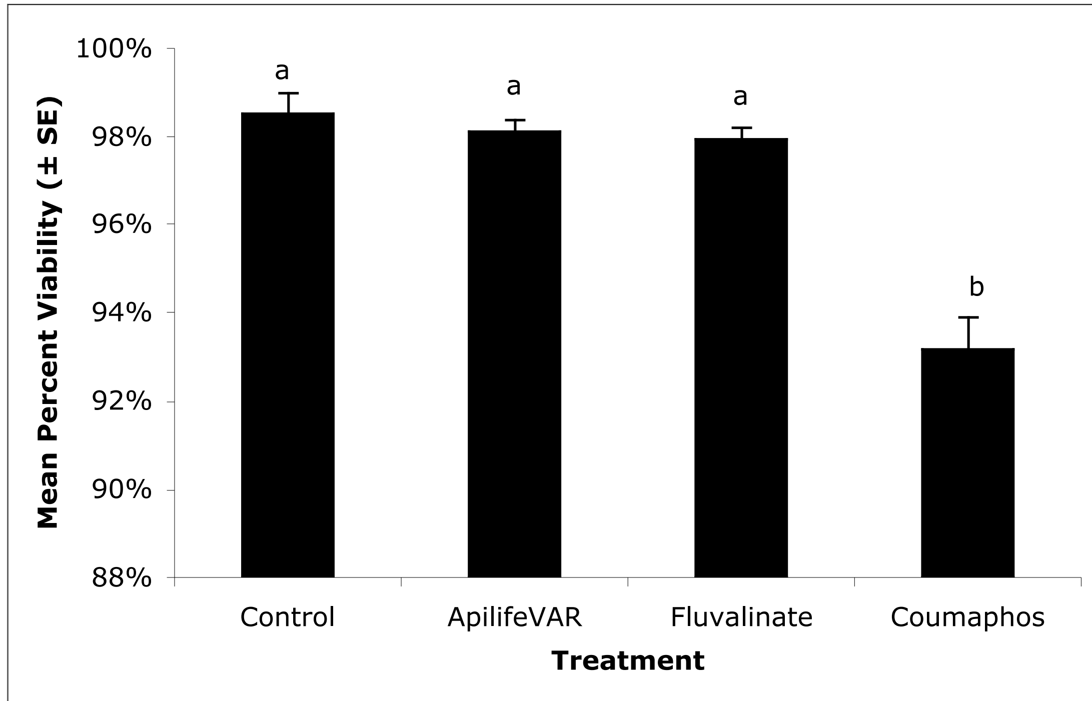

Figure. 3.1. Comparisons of the percent of live spermatozoa (mean ± SE) from drones exposed to one of four treatments (n = 16 for each treatment) during development. Sperm samples were collected at maturity from control and miticide treated hives. A one-way analysis of variance showed a significant decrease (df = 3, 60; F = 30.17; P < 0.0001) in sperm viability of coumaphos exposed drones. Columns with the same letter are not significantly different.

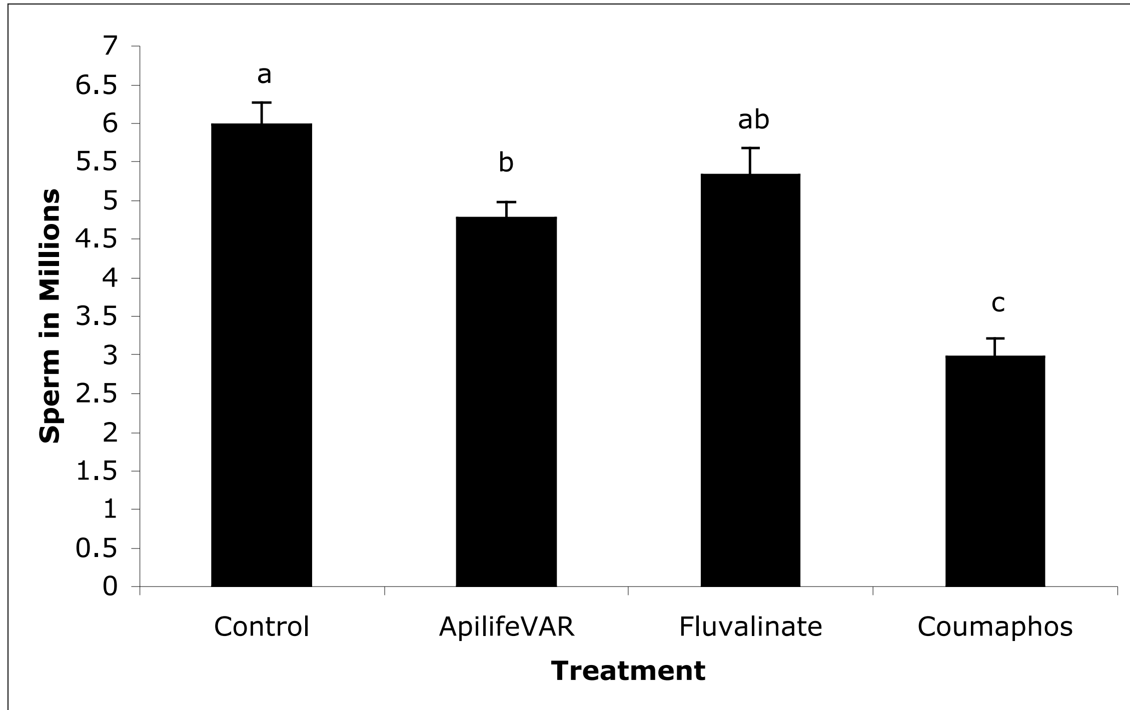

Figure. 3.2. Comparisons of total spermatozoa number (mean± SE) per seminal vesicle from drones exposed to one of four treatments (n = 16 for each treatment) during development. A one-way ANOVA yielded a highly significant difference between sperm numbers in control drones and drones exposed to thymol or coumaphos (df = 3, 60; F = 21.49, P< 0.0001). Columns with the same letter are not significantly different.

Table 3.1. Comparison of mite infestation levels in hives with differing miticide treatments measured by total mite drop during a 24 hour period. Total mite drop (mean ± SE) per colony treatment was obtained through the use of sticky board traps.

Colony treatment	**Number Colonies**	**Mean number of mites counted**
control	4	46.0 ± 4.7 *
fluvalinate	3	11.0 ± 4.6
coumaphos	3	15.3 ± 6.4
Apilife VAR®	3	12.0 ± 1.7

[1] A one-way ANOVA detected a significant difference (df = 3, 9; $F = 14.23$; $P < 0.0009$) in mite infestation in control colonies in comparison to fluvalinate, coumaphos, and Apilife VAR® treated colonies indicated by asterisk (*).

3.4. Discussion

Coumaphos treatment caused several negative effects on drone bees, including a significant decrease in spermatozoal production and viability when compared to drones of control colonies. Exposure to coumaphos during development and maturity caused a 50% reduction in the spermatozoal production and a reduction of just over 5% in sperm viability. Thymol treatment also significantly lowered sperm production in drones by approximately 20% compared to the controls.

Surviving drones in control colonies had the highest average number of spermatozoa and highest sperm viability. Drones in fluvalinate treated colonies showed no significant differences from controls. Previous studies have shown that exposure of drones to fluvalinate reduced the amount of sperm found in the seminal vesicles of drones (Fell and Tignor 2001, Rinderer et al. 1999). However, we found that fluvalinate treatment did not adversely affect sperm production or viability in exposed drones. Fell and Tignor (2001) observed an average number of $1.9 \pm 0.87 \times 10^6$ sperm per seminal vesicle of drones exposed to fluvalinate which was significantly lower than control drones with $4.3 \pm 0.64 \times 10^6$ spermatozoa. In contrast, we found an average of $5.33 \pm 0.21 \times 10^6$ sperm per seminal vesicle in fluvalinate treated drones which was not significantly different from the $5.99 \pm 0.28 \times 10^6$ spermatozoa observed in drones from control colonies. It is reasonable to speculate that our results on sperm production in drones were influenced by the death of drones before sexual maturity. Rinderer et al. (1999) observed a two thirds decrease in survival from the first day of emergence of drones reaching 12 to 18 days for drones of both control and fluvalinate treated colonies; in our study drones were collected 14 to 20 days after emergence. Perhaps only the most vigorous drones having higher sperm counts survived (De Guzman et al. 1999). Sylvester et al. (1999) found that drones which survive to sexual maturity from colonies treated with fluvalinate are equally able to mate and produce offspring as those from control colonies.

The amount of spermatozoa a drone produces varies. Woyke (1962) reported that mature drones produce between 1.50 and 1.75 μL semen containing about 11 million spermatozoa with a concentration of 7.5 million sperm per 1μL. The highest observed sperm count we observed was from drones taken from control colonies with 5.99 ± 0.28 (S.E.) $\times 10^6$ spermatozoa per seminal vesicle (or 12 million/ drone). Our observations of

sperm number are very comparable to an average of 11.9 x 10^6 spermatozoa per drone reported by Schulns et al. (2003). Schulns et al. (2003) also observed a significant decrease in spermatozoa production in smaller drones reared in worker cells as opposed to drone sized cells, which averaged 7.5 x 10^6 spermatozoa per drone, showing that sperm production in drones depends on body size. In an early study, Mackensen (1955) observed mature drones to produce an average of 10 million total sperm, which is a little low in comparison to the amount of total sperm reported by Schulns et al. (2003). Gencer and Firatli (2005) reported sperm numbers similar to both Mackensen (1955) and Schulns et al. (2003) with 12.01 x 10^6 total spermatozoa in drones from queen-right colonies compared to 10.17 x 10^6 total spermatozoa observed with drones developed from laying workers. Drones used in our study were reared in drone comb from queen-right colonies and produced 5.99± 0.28 (S.E.) x 10^6 spermatozoa per seminal vesicle, which if doubled, would mirror the 12 million total sperm observed by Gencer and Firatli (2005). Fell and Tignor (2001) reported lower numbers of sperm per seminal vesicle of control drones averaging 4.3 ± 0.64 (S.E.) x 10^6 spermatozoa. Studies on spermatozoa number per drone ranged from 4 x 10^6 to 12 x 10^6.

Several studies have been conducted on the toxicity of thymol to larvae and adult honey bees, but the effects of thymol on drone reproductive physiology is unknown. Thymol treatments seemingly have no adverse effects on adult mortality, however in young larva (0-3 days old) mortality is increased with its use (Mattila et al. 2000). The cause of toxicity of thymol to honey bees is a result of the timing and method of the thymol application. Ideally colonies should be treated with thymol when daily temperatures are between 15 and 20ºC (Mattila et al. 2000). If temperatures are below this range, thymol will vaporize too slowly and is thus ineffective in mite control. However, temperatures above this range cause rapid volatization and a significant increase in bee mortality (Chiesa 1991, Gal et al. 1992). The results of this study show a significant decrease in sperm production of drones exposed to Apilife VAR® which may be attributed to daily temperatures peaking well above the recommended threshold. The average temperature during our June and July treatments with Apilife VAR® was 19.5ºC and 22ºC respectfully, which is very close to the recommended limit for use, but during that two month period temperatures peaked at 32.7ºC, and fell to 8.3ºC, extremes which

may affect Apilife VAR's efficacy and toxicity to honey bees. It has been suggested by Mattila et al. (2000) that a gel formulation of thymol may result in more controlled release of vapors, overcoming complications of temperature dependency and reducing toxic effects on bees which oil and powder formulations of thymol may cause. It is uncertain if the lowered sperm counts of Apilife VAR® treated drones we observed were a result of toxic exposure due to temperatures well above the recommended threshold or to the inherent toxicity of the plant oils. However, the high temperatures may have been a contributing factor.

The presence, and infestation levels, of varroa mites in treatment and control colonies is a factor that cannot be ignored. Previous studies have documented negative effects of varroa infestation on drone reproductive physiology. Schneider (1986), for example, found a correlation between the intensity of varroa mite parasitization of drone brood and a reduction in the size of seminal vesicles and mucus glands. The amount of spermatozoa produced by drones decreased by 50% when drone pupae were infested with more than 3 mites compared to drones infested with 2 mites or less. However, a later study conducted by Rinderer et al. (1999) reported minor negative effects on drone weights, mucus gland and seminal vesicle weights, and the number of spermatozoa produced by drones parasitized by varroa mites, but no significant decline in spermatozoa numbers were noted. Collins and Pettis (2001) also found no decrease in semen quality in drones infested with varroa mites. They observed similar values in volume, concentration, and viability of sperm for both mite infested and non-infested drones.

It is not feasible to have completely mite free colonies for research as we have yet to find a treatment or control method to completely eradicate parasitic mites of the honey bee. All colonies used in this study had some level of mite infestation. The greatest concern is mite population levels in the control colonies. All colonies used in this experiment were young colonies placed on all new combs and re-queened in the early spring. New comb was used in all colonies to avoid effects of chemical residues from previous miticide treatments. While test colonies received fluvalinate, coumaphos, and Apilife VAR® treatments to reduce varroa mite populations, control colonies did not receive any treatment for mite control. Varroa mite drops in control colonies averaged 46 ± 4.67 (S. E.) mites per day which is at or just below the treatment threshold

recommended in the state of Virginia. Mite levels were significantly higher (P<0.0009) in control colonies than colonies receiving mite control treatments as one would expect. Despite higher mite concentrations, we observed the highest sperm viability and production in control colonies over fluvalinate, coumaphos, or thymol treated drones, leading us to believe that the severity of varroa mite infestation levels we observed was not high enough to be a factor in this study. Our findings are similar to those reported by Collins and Pettis (2001) demonstrating that varroa mite infestation does not have an effect on drone sperm viability.

Considerable variation in the viability of sperm collected from individual drones has been observed (Collins and Pettis 2001). Collins and Pettis (2001) reported viability ranging from 83- 100% for sperm samples collected from individual drones. Our observations were similar and ranged from 88.1- 99.9% viable sperm. The manner in which semen is collected for analysis is a significant factor in drone sperm viability (Collins 2004a). Collins (2004a) found that taking sperm directly from the seminal vesicle yielded the highest sperm viability of 79.3% ± 14.3 (S.E.) followed by washing semen from the endophallus of an ejaculated drone with viability averaging 77.5% ± 12.3 (S.E.). Our lowest observed mean sperm viability was 93.2% ± 0.7 (S.E) from drones of coumaphos treated colonies, which was higher than both the washing technique and seminal vesicle extraction technique used by Collins (2004a).

Queens containing low sperm viability in the spermatheca would be expected to become drone layers more quickly than well mated queens (Collins 2000b). However, Collins (2004b) observed that queens inseminated with 50% viable sperm can function as well as a fully mated queen for one season. It is uncertain whether queen performance would be affected by the significant decrease in sperm viability we observed with drones reared in coumaphos conditions, as viability was still quite high at 93.2% ± 0.7 (S.E). Further studies involving insemination of queens with coumaphos exposed drones could give a better idea of the possible effects coumaphos has on the viability of sperm stored in the spermatheca of queens, as well as her performance over time. In addition, semen in our study was examined directly after ejaculation; it is unknown how exposure of drones to miticides during their development can affect the viability of stored drone semen over time. The collection and storage of honey bee spermatozoa is commonly practiced for use

in artificial insemination and the value of stored semen depends on whether it can be used in artificial insemination to generate productive queens.

The production of properly mated queens depends on the quantity and quality of sexually mature drones. Incomplete fertilization of queens can result from an insufficient supply of mature drones and thus increase the potential number of queen failures later in the season (Roberts 1944). Deficiencies in drones could reduce the sperm contribution to the queen's spermatheca, if the queen mates with insufficient drones. The reduction of sperm production in drones as a result of thymol and coumaphos treatments could further reduce the amount of sperm actually entering the queen's spermatheca compared to normal drones (Sylvester et al. 1999). Drone deficiencies could be a contributing factor to increasing problems of queen supersedure and failure that we are encountering in southwest Virginia and throughout the United States.

4. The Effects of Fluvalinate and Thymol (Apilife VAR®) on the Viability and Amount of Spermatozoa contained in the Spermatheca of Queen Honey Bees (*Apis mellifera* L.)

4.1. Introduction

Problems associated with maintaining productive queens in the colonies have coincided with the increased use of chemical miticides used to control parasitic mites *Varroa destructor* (Truman & Anderson) and *Acarapis woodi* (Sanford 2001). Beekeepers have seen problems such as rapid queen supersedure following the introduction of a new queen, as well as an inability of colonies to naturally re-queen themselves (Sanford 2001).

Research conducted by Sokol (1996) and Currie (1999) has demonstrated that queens exposed to fluvalinate are linked to problems such as queen loss, supersedure, and queen mortality. Fluvalinate, a pyrethroid chemical sold under the trade name Apistan® , was the only registered compound in the United States for varroa mite control until 1998 (Elzen and Westervelt 2002). However fluvalinate accumulates in beeswax and remains at significant levels in colonies post treatment (Wallner 1995). The accumulation of fluvalinate in the wax leads to the prolonged exposure of bees to the chemical and can cause problems with queen oviposition as well as queen loss in treated colonies (Sokol 1996). Currie (1999) found that exposing queens to an Apistan® fluvalinate queen tab (1% formulation) reduced the queen's ability to suppress supersedure following introduction into a colony. Queens exposed to the tab for 3 days, which is the minimum exposure time recommended on the label, suffered higher supersedure rates than control queens, and only 29%(4 of 14) queens survived to the end of the summer season. Exposure to the tab for up to 7 days resulted in significant mortality of queens (6 out of 15 queens died).

In 1998 an organophosphate compound, coumaphos, was registered for varroa control due to the increasing resistance of mites to fluvalinate (Elzen and Westervelt 2002). The increased use of coumaphos has led to concerns that it may have negative, non-lethal effects on queens reared in coumaphos treated colonies (Haarmann et al. 2002). Coumaphos has been quite effective in reducing varroa mite infestation levels, but

it has had a distinct negative effect on the ability of colonies to rear queens. Haarmann et al. (2002) found that developing queens exposed to as little as one coumaphos-impregnated strip suffered a high mortality rate after 24 hours of exposure. In addition, queens that completed development showed sub-lethal effects such as lowered body and ovary weights, and lowered mean number of sperm contained in the spermatheca after mating. The use of coumaphos also affects acceptance levels of queen cells in colonies. Fell and Tignor (2001) reported at 90% decrease in acceptance of queen cells reared in coumaphos colonies compared to queen cells of control colonies.

Pettis et al. (2004) took a closer look into the potential effects of coumaphos residues on the ability of colonies to rear queens by mixing varying levels of coumaphos into wax cells used for queen rearing. They found that queen cups containing 1000 mg/kg coumaphos led to a 100% rejection by the colony, whereas a 50% rejection rate was observed with cups containing 100 mg/kg, which is the coumaphos tolerance level in beeswax set by the U.S. Environmental Protection Agency (EPA). They also observed a significant decrease in the weight of queens that were able to survive development in wax cells containing 100 mg/kg coumaphos. Collins et al. (2004) continued on to find that the presence of coumaphos in queen cells reduced the number of functioning queens in colonies by as much as 75% after 6 months.

Haarmann et al. (2002) reported a reduction in sperm stored in the spermatheca of queens exposed to coumaphos. Their findings are troubling as the supply of spermatozoa a queen has throughout her life is limited to those she obtained during mating. Once a queen has started laying, she will not mate for the remainder of her life. After mating, a queen will have an average of 80-90 million spermatozoa in her lateral oviducts (Koeniger 1986) with as many as 200 million possible (Woyke 1989). After returning to the hive, the spermatheca is filled, using about 5% of the total sperm contained in the oviducts; with less than 1/10 of the sperm from any one drone reaching the spermatheca (Koeniger 1990a). After the queen starts laying eggs, the amount of sperm held in the spermatheca continues to decrease progressively until she is superseded or becomes a drone layer (Harbo 1979a, Szabo and Heikel 1987). If the spermatheca is not sufficiently filled, then seemingly good queens may become unproductive queens (Roberts 1944).

The lowered sperm counts in spermatheca of coumaphos-treated queens observed by Haarmann et al. (2002) and the importance of healthy abundant sperm for productive queens was the driving force for conducting this study. The objectives of this study were to rear queens in colonies treated with recommended labeled concentrations of fluvalinate (Apistan®), coumaphos (Check Mite+), and Apilife VAR® (74.1% thymol, 16.0% eucalyptus oil, 3.7% l-menthol) to investigate the potential effects of these miticides on the ability of queens to store spermatozoa and maintain its viability. We were interested not only in sperm concentration in the spermatheca, but also the viability of sperm stored in the spermatheca of miticide exposed queens. In addition to coumaphos, we were also interested in other commonly used chemicals for mite control, fluvalinate (Apistan®), and Apilife VAR®, a natural oil blend, which has recently become available in the United States for mite control.

4.2. Materials and Methods

4.2.1. Queen Rearing

Four strong colonies were each crowded down into one standard full depth body and the queens were removed to meet the requirements of a starter colony for queen cell production. Each starter colony was randomly assigned a treatment of Apilife VAR®, fluvalinate, coumaphos, or control. Miticide treatments were applied to the colonies following the dose according to the label. Apistan® and Checkmite+ (10% fluvalinate or coumaphos in plastic strips) were administered to the colonies using two strips, one placed next to the queen rearing cells. The strips were left in the colony for several weeks, but were not needed for the full 6 week treatment duration. Apilife VAR®, formulated in floral foam tablets, were broken into four pieces, and enclosed in #8 mesh wire screen to prevent chewing and removal of the product by bees. The screened treatment was then placed on the top corners of the hive body. Apilife wafers were replaced with fresh wafers every 10 days. All larvae were grafted from a single colony containing a young, well-mated queen. Larvae were grafted into wax queen cell cups using standard queen rearing techniques (Laidlaw and Page 1997). Sixteen to twenty queen cell cups were attached to a cell bar and inserted into an empty frame. The empty frame was then inserted into starter colonies for queen cell production.

Sixteen 5-frame mating colonies (nucs) were established from existing strong colonies in the apiary, all with newly drawn comb. Each mating nuc was randomly assigned a treatment and was given half of the labeled dose for each miticide, as two strips is the full dose for a ten frame colony. Ten days after grafting the queen cells were removed from the starter colonies and were placed in groupings of two cells into the 5-frame mating nucs containing the same miticide treatment as the starter colony in which they were reared. Queen cells were placed in groupings of two cells to insure that at least one queen emerged in the case that one cell was not viable. Queens emerged from the cells and were allowed to mate freely. After the mated queens were observed to lay eggs (7-10 days after emergence) the queens from each treatment were removed from the mating colony, placed in a queen cage with several attendant workers, and taken into the lab for analysis. A total of eight queens were used per treatment and control except for coumaphos treatments, as no viable coumaphos exposed-queens were obtained for analysis.

4.2.2. Evaluation of Sperm Viability

Queens from all treatments were dissected, the spermatheca isolated and the tracheal net removed by gently rolling the spermatheca between the thumb and forefinger. Individual spermathecae were placed on the inside of a centrifuge tube cap. Spermathecae were punctured with sharp forceps and carefully torn open to expel the spermatozoa. The forceps were rinsed in the centrifuge tube containing 400μl of modified Kiev solution (MKS) (Kaftanoglu and Peng 1984) containing 0.05% streptomycin sulfate (Locke et al. 1990). The cap containing the punctured spermatheca was then closed and the centrifuge tube was inverted several times to insure equal distribution of sperm throughout the sample.

Sperm viability was assessed using fluorescent stains (Locke et al. 1990). Stain stock solutions were composed of 0.9% NaCl and either 500 μg/ml propidium iodide (Sigma Chemicals, St Louis, Mo.) or 1000 μg/ml Hoechst 33342 (H342) (Sigma Chemicals, St Louis, Mo.) (Locke et al. 1990). From the diluted sperm solution (400μL), 100μL was removed and combined with 100μL of a working stain solution, (950μL MKS, 10μL H342 solution, and 40μL propidium iodide solution). The sperm/stain solution was incubated at room temperature for 15-20 minutes. Sperm samples were

counted directly after the 15-20 minute incubation time to obtain the highest possible sperm viability, and to reduce inconsistencies between samples. Propidium iodide enters dead cells staining them red while H342 adheres to nucleic acids fluorescing green. A 15μL aliquot of the sample was placed on a glass slide with a cover slip, and observed under 40X magnification on a Nikon Eclipse E600 Epi-fluorescent microscope equipped with a BV-2A filter. Each sample, representing one queen, was counted 4 times using 200 sperm for each count replication. A total of 8 queens were sampled for each treatment (control, fluvalinate, and Apilife VAR®) totaling 24. In each count the sperm was scored live or dead based on the color of fluorescence. The mean percentage of live sperm for the four counts was used for statistical analysis.

4.2.3. Evaluation of Sperm Numbers

Samples used in determining sperm viability in queens were saved and used for estimation of sperm concentration within the spermatheca. A 50μl diluted sperm sample was diluted further with additional MKS for ease of counting. Four aliquots of diluted sperm were counted per sample. Total sperm number estimates were made using a Bright-Line Hausser Scientific hemacytometer and an Olympus BX40 light microscope equipped with phase-optics. A mean count of sperm was determined for each queen and a final concentration of spermatozoa contained in the spermatheca was calculated using the formula:

Number of sperm per spermatheca = average sperm count per square * 25 * 10 * dilution factor

4.2.4. Statistical Analysis

All experiments were set up as a complete randomized design. All statistical analyses were computed using SAS 9.1 software package. Prior to analysis a Shapiro-Wilk test was performed to check for deviation from normality of the samples. Both sets of data were analyzed using a one-way analysis of variance (ANOVA). A Fisher's LSD test was performed to make pairwise comparisons among treatment means. An arcsine transformation was used on all sperm viability to meet the assumptions of the analysis of variance model.

4.3. Results

Sperm Viability

The values for mean percent live spermatozoa in *Apis mellifera* queens are shown in Figure 4.1. The viability of sperm from all 24 queens examined varied from 83.0% to 95.9%. The mean sperm viability varied from a low of 86.4% ± 0.8 (S.E.) in queens exposed to Apilife VAR®, to a high of 91.7% ± 0.9 (S.E.) for queens reared in control conditions. The Shapiro-Wilk test did not reveal any significant departure from the normal distribution ($W = 0.974059$; $P< 0.7667$). Exposure of queens to miticides during development was a significant factor in sperm viability ($df = 2, 21$; $F = 8.90$; $P < 0.0016$). Sperm viability in queens exposed to Apilife VAR® during development was significantly lower than the sperm viability of control queens or queens exposed to fluvalinate (Figure 4.1). Rearing queens in colonies treated with fluvalinate had no significant effect on the viability of sperm stored in the spermatheca. No viable queens were obtained from queen cells reared in coumaphos treated colonies for measuring sperm viability or spermathecal sperm numbers.

Numbers of Spermatozoa

The values for mean sperm content of the spermatheca of queens are shown in Figure 4.2. Sperm numbers ranged from 3.65×10^6 to 6.97×10^6 spermatozoa per spermatheca. A mean sperm number of 6.29 ± 0.26 (S.E.) $\times 10^6$ was found for the control queens and the treatment means for fluvalinate, and Apilife VAR® were 5.35 ± 0.38 (S.E.) $\times 10^6$, and 5.14 ± 0.38 (S.E.) $\times 10^6$ spermatozoa respectively. Exposure of queens to miticides during development was not a significant factor in the amount of sperm held in the queen's spermatheca ($df = 2, 21$; $F = 3.10$; $P < 0.0663$). No decreases in spermatozoa numbers were observed in queens exposed to fluvalinate and Apilife VAR® compared to control queens (Figure 4.2).

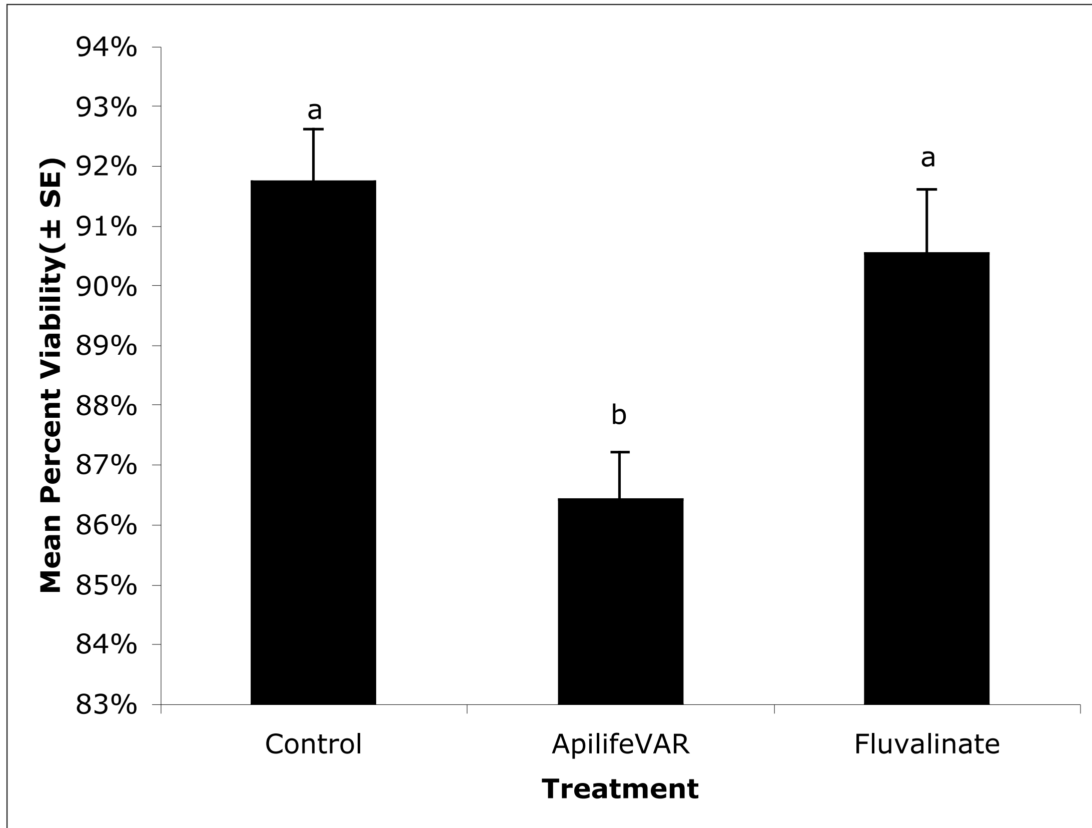

Figure. 4.1. Comparisons of the percentage of live spermatozoa (mean ± SE) from queens exposed to one of four treatments (n = 8 for each treatment) during development. A one-way analysis of variance showed a significant decrease (df = 2, 21; F = 8.90; P < 0.0016) in sperm viability of queens exposed to Apilife VAR®. Columns with the same letter are not significantly different.

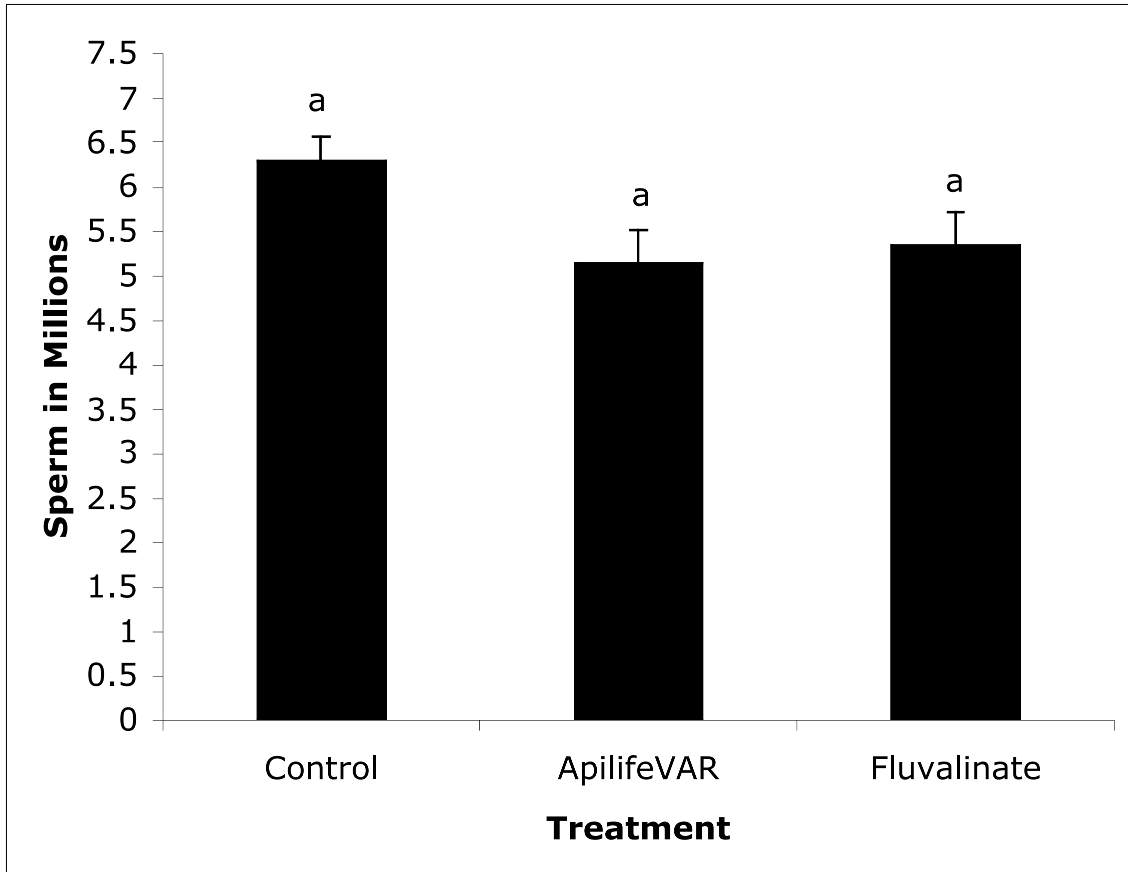

Figure. 4.2. Comparisons of total spermatozoa number (mean± SE) per spermatheca of queens exposed to one of four treatments (n = 8 for each treatment) during development. A one-way ANOVA did not yield a significant difference between treatments (df = 2, 21; F = 3.10; P < 0.0663). Columns with the same letter are not significantly different.

4.4. Discussion

Several attempts were made to rear queens in coumaphos treated colonies without success. The failure to rear queens in colonies inoculated with coumaphos was not surprising given the previous research by Haarmann et al. (2002), Pettis et al. (2004), and Collins et al. (2004), all of whom documented high mortality rates for queens reared under coumaphos treated conditions.

In this study, exposure of developing queens to coumaphos seemed to prevent queens from reaching the pupal stage of development. When queen cells reared with coumaphos failed to emerge, the cells were opened. Most cells opened contained only small amounts of royal jelly and no pupa. Some cells also contained a small larva presumed to be dead. Pettis et al. (2004) and Collins et al. (2004) found that grafted larvae were either removed by workers before capping, or the cells were torn down during the queen rearing process due to death of the larvae. However, all capped mature queen cells on day 10 post grafting contained a mature pre-emergent queen pupa. Haarmann et al. (2002) observed that any capped queen cell remaining in a starter colony inoculated with coumaphos was either dead or abnormally developed, which agrees with our observations. Collins et al. (2004) reported retardation in the development of queens exposed to coumaphos based on cuticular color of the pre-emergent pupae. Our observations were dissimilar in that no pupal-stage queens were found in capped cells indicating that death of the larvae occurred, not slowed development.

Although a few queen cells were capped over by the starter colony, no adult queens ever emerged from any of the queen cells. Roughly one half of grafted larvae were rejected by the starter colony. Fell and Tignor (2001) reported even higher rates of rejection at greater than 95% with queens grafted into coumaphos treated colonies compared to controls. Worker bees may sense something abnormal about the cell and remove it's contents, or direct toxicity effects from coumaphos in the wax are possible factors for larval rejection (Pettis et al. 2004). Although rejection of grafted larvae can occur when there is damage to the larvae from poor grafting (Pettis et al. 2004), queens of control, fluvalinate, and Apilife VAR® colonies were not observed to have the same problem of rejection, and were grafted in the same manner.

Haarmann et al. (2002) suggested that direct toxicity of larvae to coumaphos occurs from contact of nurse bees that are carrying the chemical on their bodies and transfer it to the larvae. Alternatively the chemical is incorporated into the queen cell wax as it is being constructed. Haarmann et al. (2002) found that larvae reared in vertical hanging queen cells versus the horizontal worker cells had higher mortality rates. New wax is secreted for queen cell production, and thus a higher chance for coumaphos incorporation. Workers, on the other hand, are reared repeatedly in the same cell with little to no new wax added. Their results indicated that the probable death of larvae reared in queen cells is due to higher concentrations of coumaphos incorporated into new wax. In our study, at least one coumaphos strip was placed directly next to the developing queens and thus high levels of coumaphos were likely transferred to the larvae, both by contact with nurse bees and from the production of new wax for queen cell development.

The development of queens in miticide treated colonies had no effect on the total amount of sperm contained in the spermatheca of queens. Haarmann et al. (2002) observed lowered sperm counts of spermathecae of high-dose fluvalinate exposed queens compared to control and low-dose queens, but a significant difference was not documented. In our study, Apilife VAR® exposed queens, not fluvalinate treated queens, had the lowest of the three sperm counts averaging 5.14 ± 0.38 million sperm, but no significant differences were observed. Previous studies conducted by Harbo (1979a) and Woyke (1962) have shown means of 5.00 ± 0.26 million and 5.34 ± 0.12 million sperm respectfully in the spermatheca of queens at onset of oviposition, which are similar to our observations. A higher spermatozoa average of 9.77 ± 0.97 million has also been found in the spermathecae of newly mated queens (Szabo and Heikel 1987). However, we did not observe such high sperm numbers, with our highest number being that of a control queen with 6.97 million sperm contained in the spermatheca.

Fluvalinate has been shown to have minor negative effects on seminal vesicle weights (Rinderer et al. 1999), and significantly decrease spermatozoa production in drones exposed to the chemical during development (Fell and Tignor 2001). Because our queens were openly mated, we cannot be certain about the quantity of sperm our queens received during mating. A single drone will produce more sperm than a queen's spermatheca can hold (Koeniger 1990b) and less than 10% of the sperm from each drone

reaches the spermatheca (Koeniger 1990a). With a queen mating with anywhere from 6 to 24 drones (Woyke 1962, Winston 1991, Neumann et al. 1999) it is not surprising that the amount of sperm contained in the spermatheca was not significantly changed by the queen's exposure to miticides. These results indicate that reduction in spermathecal sperm numbers are most likely due to problems with drone deficiencies and not to sub-lethal effects of miticides on queens. A study involving the instrumental insemination of queens with drones from a miticide treated colony, currently underway, could give us a better idea of the impact miticides could have on sperm storage in the spermatheca.

Viability of sperm contained in the spermatheca of queens is negatively affected by exposure of the queen to Apilife VAR® during development. The Apilife VAR® thymol-blend treatment lowered sperm viability by just over 5% compared to control queens ($P < 0.0016$). Colonies should be treated with Apilife VAR® when daily temperatures are between 15 and 20ºC (Mattila et al. 2000). Temperatures above this range cause rapid volatization and a significant increase in bee mortality (Chiesa 1991, Gal et al. 1992). The significant decrease in sperm viability in the spermatheca of queens exposed to Apilife VAR® observed in our study may be attributed to an high volatility and increased toxicity as daily temperatures peaking well above the recommended threshold for safe use. The average temperature during our June and July treatments with Apilife VAR® was 19.5ºC and 22ºC, respectfully, which is very close to the recommended upper temperature limit for its use. However, during that two month period temperatures peaked at 32.7ºC. The upper extreme may have affected the efficacy and toxicity of Apilife VAR® to the honey bees. It has been suggested by Mattila et al. (2000) that a gel formulation of thymol may result in more controlled release of vapors overcoming complications of temperature dependency and reducing the toxic effects on bees that oil and powder formulations of thymol may cause.

In our study, wax used to make queen cups for grafting was made from un-contaminated wax. Testing the concentration of miticides found in the wax of mature queen cells would have been helpful in understanding the level of miticides at which the grafted larvae were exposed. Haarmann et al. (2002) collected wax samples from queen cells from 5-frame nucs inoculated with either fluvalinate or coumaphos. They found that queen cells in nucs exposed to fluvalinate contained from 2.28 mg/kg in a high dose

group (twice our dose) to 3.70 mg/kg fluvalinate in the low dose exposure (half our dose). Queen cells exposed to coumaphos contained much higher miticide concentrations of 181 kg/mg and 237 kg/mg in the low dose and high dose exposures respectfully.

A queen releases many more sperm than necessary for successful fertilization of the egg, estimated between 20-35 spermatozoa (Harbo 1979a). Collins (2000a) suggested that the ratio of worker to drone pupae in a colony may not necessarily reflect the proportion of live spermatozoa in the spermatheca of queens. Collins (2000a) found that queens inseminated with differing levels of viable sperm (65.1%, 55%, 42.6%, and 32.2%) shifted the amount of dead sperm held in the spermatheca as all sperm mixes had similar levels of sperm viability contained with in the spermatheca 27 days post insemination. Queens that received 42.5% live sperm or higher produced all worker brood suggesting that semen only needs to be at levels of 42.5% viability or higher to have successfully mated queens as live spermatozoa in spermatheca of those queens averaged 61% (Collins 2000a).

In a later study, Collins (2004b) found that queens inseminated with 50% fresh semen consistently laid all fertilized eggs 6-8 months post insemination demonstrating the functional longevity of queens inseminated with low viable sperm. Based on Collins' (2000a and 2004b) observations, the significant drop we observed in sperm viability of spermatozoa contained in the spermatheca of Apilife VAR® exposed queens should not affect queen performance. However, it is unclear what the long term effects of Apilife VAR® or any other miticide on the sperm viability of spermatozoa held in the spermatheca as well as brood patterns of exposed queens. Queens in our study were dissected and analyzed days after they started to lay. It is possible that the viability levels of 86.4% ± 0.8 (S.E.) we observed in queens exposed to Apilife VAR® could be drastically lower in 2-3 months time if queens were continually exposed to the miticide. Queens exposed to fluvalinate did not show a decrease in viability of sperm contained in the spermatheca immediately after egg lay, however, it is possible that changes in viability of sperm held in the spermatheca of fluvalinate exposed queens could lower with long term exposure.

Miticides should be used sparingly to avoid possible effects on queen reproduction as high levels of pesticides in beeswax may significantly reduce queen

performance (Collins et al. 2004). Beekeepers should rotate old combs out of colonies to reduce the impact of miticide accumulation in the wax (Pettis et al. 2004). Deficiencies in miticide exposed drones, such as lowered sperm production and viability, could possibly reduce the amount of sperm entering the queen's spermatheca (Sylvester et al. 1999); a problem which has been speculated to cause early supersedure of queens (Camargo and Gonclaves 1971). Our study did not show an effect of miticide exposure of queens on the amount of actual sperm stored in the spermatheca, but additional studies involving insemination using miticide exposed drones could further our understanding of the role miticides may play in the problem of maintaining productive queens. The use of Apilife VAR®, and other thymol blends should be limited in colonies used for queen production as lowered viability of spermatozoa in the spermatheca resulted from its use.

5. Survival of Honey Bee (*Apis mellifera* L.) Spermatozoa Incubated at Room Temperature from Drones Exposed to Miticides

5.1. Introduction

Honey bees, in addition to most ant species, are unique in the fact that spermatozoa live for several years in the spermatheca of queens in brood nest temperatures of 35ºC (Collins 2000b). Queens mate between 5 and 8 days of age and store the received sperm at that time for the rest of their reproductive lives. The lengths of time honey bee spermatozoa are able to survive make collection and storage of honey bee semen possible for use in artificial or instrumental insemination. The success of artificial insemination is highly dependent on the quality of the sperm used (Peng et al. 1990).

In recent years, problems have arisen with queen performance in the hive. Beekeepers have noted problems with supersedure, following the introduction of a new queen, as well as an inability of colonies to naturally re-queen themselves (Sanford 2001). The increasing problems associated with maintaining productive queens have coincided with increased use of chemical miticides for parasitic mite control (Sanford 2001). Miticide use in colonies had shown deleterious effects on queen health and reproductive physiology. Several articles (Collins et al. 2004, Pettis et al. 2004, Haarmann et al. 2002, Fell and Tignor 2001) report findings of negative effects of coumaphos on queens including high mortality, lowered body and ovary weights, decreased (mean) sperm numbers held in the spermatheca, lowered acceptance of larvae grafted for queen cells, and reduced numbers of queens functioning in colonies by 75% after 6 months.

Miticide use in honey bee colonies has also been shown to reduce drone survival (Rinderer et al. 1999) and production (De Guzman et al. 1999), body and mucus gland weights (Rinderer et al. 1999), and spermatozoa production (Fell and Tignor 2001). However, the effects of miticides on the viability of sperm produced by drones exposed to miticides during development are unknown. It is possible that decreasing sperm viability due to exposure of drones to miticides plays a role in the current problems associated with queen performance. Queens containing sperm with low viability in the

spermatheca would be expected to become drone layers more quickly than well mated queens (Collins 2000a).

The age of the drone at the time of sperm collection may have an effect on insemination success. Woyke and Jasinski (1978) observed lowered numbers of sperm entering the spermatheca of artificially inseminated queens as drone age at the time of collection increased. Observations made by Locke and Peng (1993) support Woyke and Jansinski's (1978) findings as they saw a significant decrease in sperm viability of drones 4 and 6 weeks of age compared to fully matured 2-week-old drones. The time of year may also play a role in insemination success as Kumar and Kaur (2003) observed higher spermatozoa production of drones in the spring compared to the fall. Also higher carbohydrate and proteins, which provide nutrition for sperm during storage, were found in semen when collected in the spring.

Rapid decreases in viability of sperm contained in the spermatheca of queens obtained from mating with miticide exposed drones could lead to queen loss and supersedure. It is important to consider the possible effects of miticides on the viability of exposed drones with regards to queen health and performance in the colony. The objectives of this study were to rear drones in colonies treated with recommended labeled concentrations of fluvalinate (Apistan®), coumaphos (Check Mite+), and Apilife VAR® (74.1% thymol, 16.0% eucalyptus oil, 3.7% L-menthol) to investigate the potential effects of these miticides on the viability of sperm as measured by their ability to survive *in vitro* storage over a 6 week period.

5.2. Materials and Methods

5.2.1. Drone Rearing

Drones were produced in standard 10 frame colonies with new untreated combs. Naturally mated sister queens grafted from the same mother were placed in each standard full depth hive. Each colony was given a frame of drone comb to heighten drone production. Colonies were checked periodically starting in early February. As soon as queens started laying unfertilized eggs for drone production, treatments were placed in colonies. A total of 12 colonies were used in the experiment, 3 for each treatment and control. Treatments consisted of either thymol (Apilife VAR®), fluvalinate (Apistan®), or

coumaphos (Checkmite +) and were assigned randomly to colonies. All treatments were administered at the labeled dose.

Apistan® and Checkmite+ (10% fluvalinate or coumaphos in plastic strips) were administered to the colonies using two strips, one of which was placed next to the drone comb. Plastic strips were left in the colony for 6 weeks and were replaced a second time at the end of the first 6 weeks. Apilife VAR® tablets, formulated in floral foam, were divided into four equal size pieces, enclosed in #8 mesh wire screen to prevent bee chewing and removal, and placed on the top corners of the hive body. Apilife wafers were replaced with fresh wafers every 10 days. Mature drones were collected in late afternoon from colonies using an entrance drone trap. Traps were set at all three colonies of a treatment for drone collection. Roughly 25 drones were collected from each of the three colonies and were then mixed creating a pool of drones (Figure 5.1). Drones used for semen collection were randomly selected from the drone pool to reduce the between hive effect. Drones that were not used immediately were held overnight in cages made with queen excluder material, allowing workers to enter and feed the drones (Collins 2004a).

5.2.2. Colony Mite Levels

At the end of the experiment varroa mite levels were estimated in treatment and control colonies using a sticky board method. A 12 x 12 inch fly trap paper was covered with black plastic mesh and placed on the bottom boards in each hive. Sticky boards were removed 24 hours after placement in the colonies and total mite counts were recorded for each hive. All 12 hives were tested for mite levels, three for each treatment and control. An average mite drop for each treatment and control was calculated.

5.2.3. Sperm Collection

Semen was collected from drones using a Latshaw instrument and syringe (Cobey and Latshaw 1998). The syringe was connected to a 50μL glass capillary tube attached to a fine glass tip. A small amount of sterile saline (sodium chloride, 0.85%; di-hydrostreptomycin 0.25%) was used as a lubricant for the syringe tip and was collected with the semen. The saline was produced from de-ionized sterilized water. Collection of semen followed procedures similar to Collins (2000b). Semen was collected from ~30 drones for each treatment. About 1.0-1.25 μL of sperm can be collect from each drone

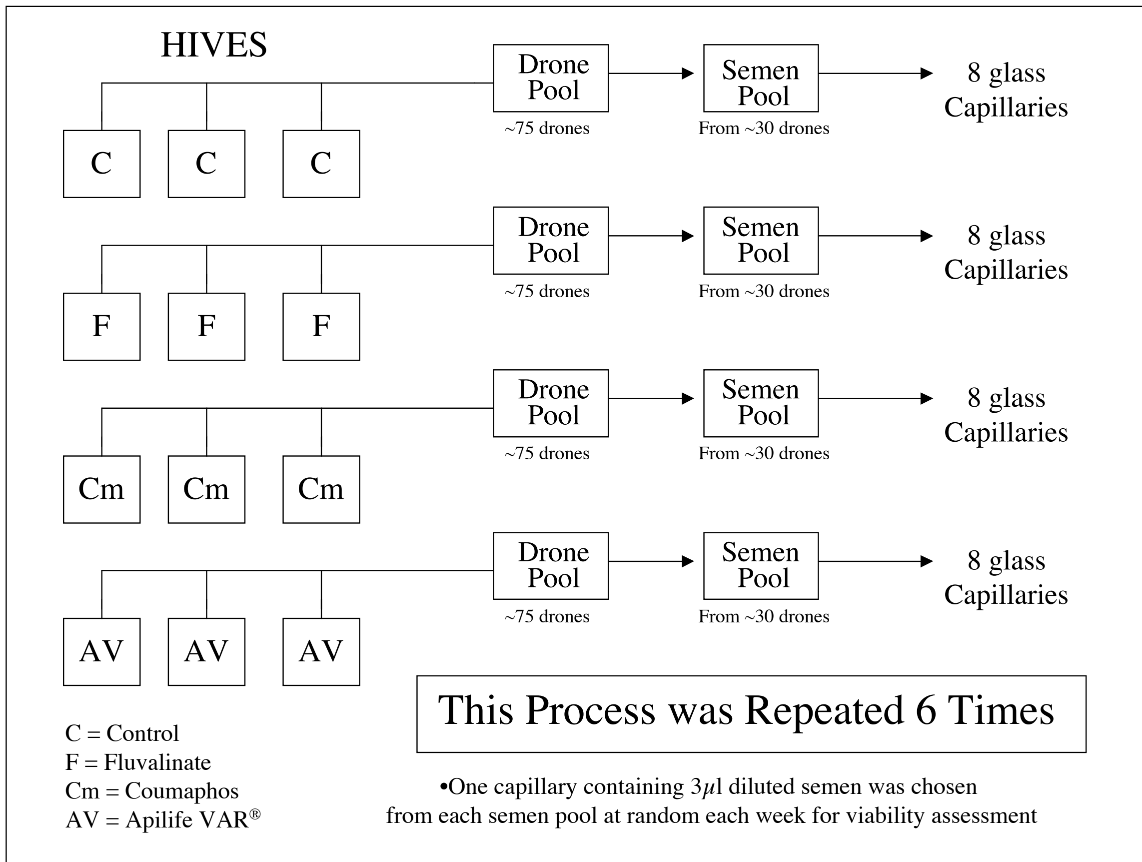

Figure. 5.1. Diagrammatic representation of the methods used for drone collection and the generation of the semen pool. The semen pools (~20μL) were diluted with an equal volume of Modified Kiev solution and then divided into glass capillaries. This process was repeated 6 times for each group of hives.

post forced ejaculation (Woyke 1985). After a sufficient amount of semen was collected (~20μL) the pooled sample of was expelled into a sterile centrifuge tube containing an equal amount of modified Kiev solution (MKS)(Kaftanoglu & Peng 1984) containing 0.05% di-hydrostreptomycin (Locke & Peng 1990), diluting the semen by half. The centrifuge tube was inverted several times to gently mix the sperm solution. The diluted semen was then recollected in sterile glass capillary tubes for storage. Six separate pools (replications) of semen were collected for each treatment and control (Figure 5.1).

5.2.4. Storage Conditions

Small capillary tubes were scored and broken from 50μL glass capillaries and sterilized. The diluted semen mixture was collected in 3μL aliquots with sterile saline into the small sterile glass capillary tubes, using the syringe. There are about 150 million spermatozoa in 20μL of collected semen (Woyke 1985). After dilution by half with MKS we would expect our 3μL sub-samples to contain roughly 11.25 million sperm. A small air bubble was placed between semen and saline on both ends of the diluted sperm to protect the sample from contamination and to help prevent the sample from drying out. Samples were further protected from drying by sealing glass capillaries at one end with Critoseal (Sherwood Medical Industries, St. Louis, MO), and the other end with petrolatum. Capillary tubes of the same pool were enclosed in a small cardboard container and the container was labeled with treatment, pool number, and date of collection. Samples were then placed in an incubator set at 25ºC (room temperature) with no light for 6 weeks. Every week following the initial collection diluted sperm samples were selected at random from each pool for viability assessment.

5.2.5. Viability Assays

Single tubes of semen of all six pooled samples for each treatment were assayed for viability immediately after semen collection, and 1, 2, 3, 4, 5, and 6 weeks following the initial collection. The capillaries were scored on each end and expelled into a 1000μL centrifuge tube containing 100μL of MKS and a 100μL working stain solution (950μL MKS, 10μL H342 solution, and 40μL propidium iodide solution). The tubes were then incubated at room temperature for 15-20 minutes. Sperm samples were counted directly after the 15-20 minute incubation time to obtain the highest possible sperm viability, and to reduce inconsistencies between samples.

A small 15 μL aliquot of the sample was placed on a glass slide with a cover slip, and observed under 40X magnification on a Nikon Eclipse E600 Epi-fluorescent microscope equipped with a BV-2A filter. Each sample, which was taken from one pooled sample, was counted 4 times using 200 sperm per count. A total of 6 samples, one from each pool, were counted for each treatment each week. In each count the sperm were scored live or dead based on the color of fluorescence. An average percentage of live sperm from the four counts of each sample was used for statistical analysis.

5.2.6. Statistical Analysis

Experiments were set up as a complete randomized design. The averages of the 6 observations per treatment from weeks 0-6 were analyzed using repeated measures analysis of variance, using PROC GLM (SAS 9.1 software). Also orthogonal contrast comparisons were made between and within treatments for each week. The comparisons of interest were between the four treatments, changes during the time of storage, and any interaction of these two factors. Means were separated by the method of least significant difference, using least squares means. An arcsine transformation was used on all sperm viability data to meet the assumptions of the analysis of variance model. Varroa mite data were analyzed using a one-way analysis of variance (ANOVA) and a Fisher's LSD test was performed to make pairwise comparisons among treatment means.

5.3. Results

Survival of Stored Sperm

The viability of sperm stored over the six week period is shown in Figure 5.2. Survival of spermatozoa stored at room temperature showed a decreasing trend over the 6 week sampling period for all three treatments and control. Exposure of drones to miticides during development was a significant factor in viability of stored sperm (df = 3, 20; F = 57.93; $P < 0.0001$). A significant interaction of treatment by time was also observed (df = 18, 120; F = 9.73; $P < 0.0001$). The survival of sperm stored from drones exposed to coumaphos was significantly different ($P < 0.005$ for all weeks) from survival of samples of the other three treatments and controls for all weeks (0-6) (Figure 5.2). Contrasts between control versus fluvalinate and Apilife VAR, and fluvalinate versus Apilife VAR did not show significant differences. The initial viability of the samples of

stored sperm of coumaphos treated drones was the lowest with a mean viability of 86.2% ± 0.5 (S.E.) and continued throughout the 6 week sampling period reaching the lowest viability at the end of week 6 with 48.6% ± 2.8 (S.E.) viability. Control, fluvalinate and Apilife VAR® treatments had stored sperm viabilities within a few percent of each other through the sampling period with the highest difference of 4.14% between control and Apilife VAR® treatments in week six. No significant differences were found between them. However, significant differences were observed between weeks 5 and 6 for all treatments (Table 5.1) and for viability of the controls during week one from 90.7% to 86.8% ($P < 0.0152$).

Varroa Mite Loads

Mite counts in colonies ranged from 4 to 39 mites per colony. Mean mite counts per treatment are shown in Table 5.2. Control colonies had the highest mite drop in 24 hours averaging 22.7 ± 8.4 (S.E.) mites. Colonies treated with fluvalinate, coumaphos, and Apilife VAR® averaged 8.3 ± 3.8 (S.E), 13.67 ± 5.5 (S.E.), and 5.67 ± 0.88 (S.E.) mites respectfully. No significant differences were observed between treatments.

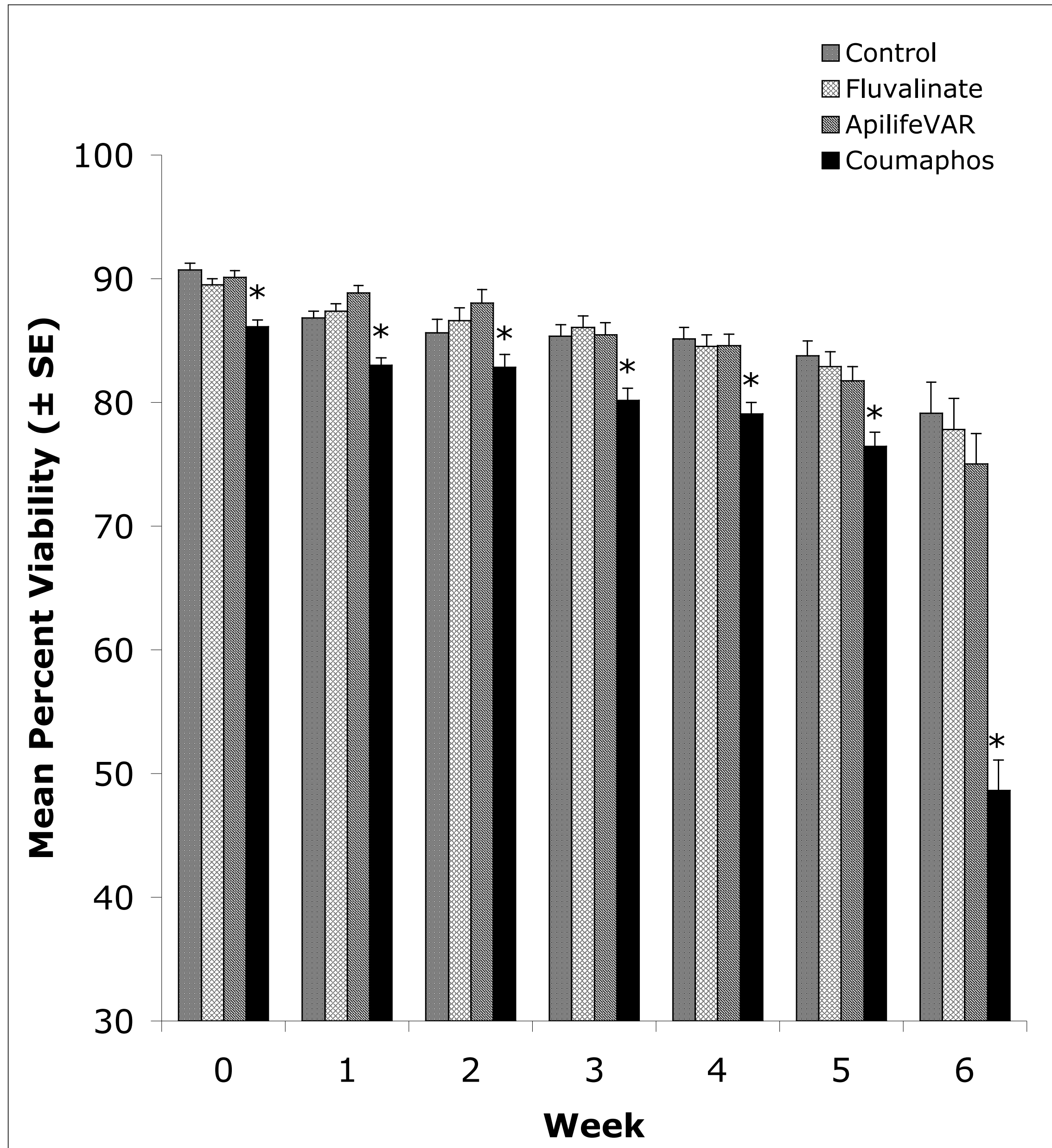

Figure. 5.2. Comparisons of the percent viability of honey bee spermatozoa from drones exposed to different treatments during development and sexual maturation. Pooled semen samples were collected from drones and stored in glass capillary tubes held at 25°C and analyzed for percent viability over a 6-week period. *Repeated measures analysis detected a significant decrease in sperm survival (df = 3, 20; F = 57.93; $P < 0.0001$) for all weeks from drones exposed to coumaphos. Significant differences ($P < 0.005$ or less for all weeks) obtained through orthogonal contracts for each week are noted by the asterisks (*) above coumaphos columns.

Table 5.1. Significant values for decreased sperm viability of stored semen from miticide exposed drones between week 5 and week 6 of storage.

Week	Treatment			
	Control	**Coumaphos**	**Fluvalinate**	**Apilife VAR®**
Change in viability from week 5 to week 6	83.8 - 79.2% $F = 9.08$ $P < 0.0048$	76.5 – 48.6% $F = 140.12$ $P < 0.0001$	82.9 – 77.9% $F = 10.27$ $P < 0.0029$	81.7 – 75.0% $F = 18.97$ $P < 0.0001$

[1] Significant differences, detected by orthogonal contrasts, were observed for each treatment and control from week 5 to week 6.

Table 5.2. Comparison of mite infestation levels in hives with differing miticide treatments measured by total mite drop during a 24 hour period. Total mite drop (mean ± SE) per colony treatment was obtained through the use of sticky board traps.

Colony treatment	**Number Colonies**	**Mean number of mites counted**
control	3	22.7 ± 8.4
fluvalinate	3	8.3 ± 3.8
coumaphos	3	13.7 ± 5.6
Apilife VAR®	3	5.7 ± 0.9

[1] A one-way ANOVA did not detect a significant difference (df = 3, 9; $F = 1.92$; $P < 0.2047$) in mite infestation between miticide treatments.

5.4. Discussion

The assessment of viability shows good survival (75- 79%) of sperm to week 6 except with sperm stored from coumaphos exposed drones. Stored semen from the coumaphos treatment had acceptable viability levels averaging 76.5% at week five, and then significantly decreased (df = 1; F= 140.12; P < 0.0001) to 48.6% viability by week six. Locke and Peng (1993) in a study looking at viability of honey bee sperm stored for 6 weeks started with and average of 86.9% viable sperm and ended with 77.6% viable sperm at week six. Our values were very similar with an initial average viability of 90.1% ending with and average of 77.4% (excluding coumaphos viability data). Collins (2000b) also found similar viability at week 6 with sperm survival between 70-80%.

In a study examining the functionality of queens inseminated with low viability sperm Collins (2000a) found that queens that received 42.5% viable sperm or higher produced all worker brood. Later she observed that queens receiving 42.5% viable sperm at insemination consistently laid fertilized eggs 6-8 months post insemination (Collins 2004b). The average viability of 48.6% observed on week 6 for our coumaphos treatment is above the 42.5% threshold that Collins (2000a, 2004b) observed. However, it is not known if, and how fast, the viability of stored sperm from coumaphos treatments would decline if it were continued to be stored in the glass capillary tube past week 6. More important is the possible effect rapid decreases in sperm viability would have on the performance of queens mated with miticide exposed drones.

Collection of drones for this study occurred in the late spring and early summer; a time that should result in high numbers of sperm produced by drones as opposed to drones collected in the fall (Kumar and Kaur 2003). Drones were also collected 14-20 days after emergence. Drones were not collected later than 20 days after emergence, as Locke and Peng (1993) reported a significant decrease in sperm viability of drones 4 to 6 weeks in age compared to drones 14 days of age. The mean (± SE) sperm viability of individual drones exposed to coumaphos during development was 93.2% ± 0.7 (see chapter 3). In this study we observed a mean (± SE) sperm viability of 86.2% ± 0.5 for the pooled sample of semen collected from drones exposed to coumaphos. At age of sexual maturity drones exposed to coumaphos during development should average 86.2-93.2% sperm viability, according to our findings. Queens that mate with mature drones

from coumaphos treated colonies would be expected to contain sperm with high viability in their spermatheca directly after mating with coumaphos exposed drones. However, the viability of sperm in the spermatheca of queens would be expected to significantly drop 6 weeks after mating, based on the results of this *in vitro* study. It is unknown how a sudden decrease in sperm viability would affect queen performance, but miticide exposure of drones seemly has latent effects on sperm viability that would inherently be expressed in queen performance over time.

Coumaphos not only decreases the viability of sperm produced by drones exposed to it, but it has also shown to cause lethal, and sublethal effects to queens including lowered mean number of spermatozoa contained in the spermatheca of queens after mating (Haarmann et al. 2002). Decreasing amounts of sperm entering the spermatheca of coumaphos exposed queens along with lowered sperm viability of exposed drones could lead to queen failure. Collins (2000a) inseminated queens with varying levels of sperm viability (32.2- 65%) and observed similar levels of live spermatozoa in all inseminated queens 27 days after insemination. Collins (2000a) speculated that when a queen is inseminated with semen with moderate viability (56-62%), most of the dead sperm are dragged along in migration into the queen's spermatheca and then removed at a later time. On the other hand with sperm that has a lower viability (32.2-42.6%), less dead sperm are dragged along because the load of dead sperm is too much for the activity level of live sperm. Unfortunately, Collins (2000a) did not provide data to support her hypothesis. It is possible that the similarities in sperm viability noted 27 days after insemination were not due to the removal of dead sperm, but rather to the fact only live sperm move into the spermatheca. The selective intake of sperm could also lead to lower numbers of sperm in the spermatheca. Determining the number and viability of spermatozoa contained in the spermatheca a few days after insemination with low viability sperm, and then at a later date, would indicate whether dead sperm are taken up by the spermatheca, or whether the queen exhibits some degree of selection in sperm uptake.

The level of varroa mites in our treatment and control colonies were evaluated due to documented negative effects varroa mites have on the reproductive physiology of drones. Varroa infestation of drones has led to such abnormalities as reduction in the size

of seminal vesicles and mucus glands, and a reduction in spermatozoa production (Schneider 1986, Rinderer et al. 1999). However, a recent study by Collins and Pettis (2001) found no decrease in semen quality in drones infested with varroa mites. No significant differences were found regarding volume, concentration, or viability of sperm for mite infested and un-infested drones.

In our study, no significant differences were found in mite levels between treatments. Control colonies had the highest mean mite drop in a 24 hour period with 22.7 ± 8.4 (S. E.) mites as one would expect. The average mite levels we observed were far below the 40-50mites/24hour threshold recommended in the state of Virginia for mite treatment. The low numbers we observed in all of our colonies may be attributed to the fact that all colonies were evaluated in the spring as opposed to later in the season when honeybee and mite populations are at their peak. With no documentation of varroa infestations negatively affecting sperm viability (Collins and Pettis 2001) and mite levels so low in our hives, it is unlikely that varroa mite feeding had an effect on our results.

The term drifting is defined as the movement of bees between colonies (Butler 1939). Drifting of drones makes it difficult to insure that drones selected from colonies for sperm collection are the progeny of that colony's queen (Currie and Jay 1991). Similarly, our concern was that the drones we collected from each colony were in fact from that colony and not a drone from a colony of a different treatment.

Neumann et al. (2000) observed on average 5.0%± 0.7 (S.E) of workers in a colony drifted compared to a significant increase in drone drifting at 50.0% ± 6.8 (S.E.). Drones begin drifting at 6-7 days of age with drifting levels as high as 50% at 15 days old (Currie and Jay 1991). The size of a colony has no correlation with the amount of drift seen with drone populations (Neumann et al. 2000). The directions that drones drift in an apiary are related to the position and movement of the sun, as well as mating status of queens in surrounding hives (Currie 1986). Currie (1986) found that of the drones that drifted, 48% drifted to colonies with virgin queens, 10% visited colonies that were queenless, and 19% visited queen-right colonies. Drone drifting behavior decreases with hives at distances of 50m apart or farther (Currie and Jay 1991). Currie and Jay (1991) looked at how arrangement of hives in an apiary affected drone drifting behavior and

found that drifting was significantly reduced between hives arranged in a pair compared to 5 hives in a row, horseshoe formations, or colored or offset entrances.

The hive placement and the methods used for drone collection in our study helped to minimize the effects of drone drift. First, drones used in our study were collected using entrance drone traps that prevent drones from leaving the colony for orientation or mating flights. If we had collected drones in the late afternoon as they were coming back from mating flights the probability of collecting drifted drones could have been higher. However, it is possible that drones collected in the drone trap could have drifted to that colony the previous day. Secondly, when drones were collected in May, all surrounding colonies contained mated queens and thus were less likely to be visited by drifting drones according to Currie (1986). Third and last, the hives used for this study were arranged in pairs, which according to Currie and Jay (1991) significantly reduce drift compared to other hive formations or strategies used to reduce drift.

In *in vitro* studies involving the storage of sperm, the concentration of cells/unit volume can play a significant role in sperm survival. A sperm storage study involving bull spermatozoa showed that the ability of sperm to survive storage after dilution was directly correlated with the number of spermatozoa in the diluted sample (Salisbury et al. 1943). A similar study involving the storage of diluted chicken semen, found that lower dilution rates of sperm gave the highest fertility (Wilcox and Clark 1962). In our study the concentration of honey bee spermatozoa per micro liter was not controlled. Since previous studies have shown that exposure of drones to coumaphos and Apilife VAR® significantly lowered sperm production (see chapter 3), it is possible that the observed differences in viability could have been due to concentration effects. Although all pooled semen samples were diluted in half by MKS, the semen samples collected from coumaphos drones may have had a lower concentration of sperm / µL compared to the other treatments, a factor that could have played a role in sperm survival over time. The increased availability of oxygen to the spermatozoa has been suggested as a cause behind the decreased survival of spermatozoa over time with increased dilution (Shannon 1965).

The study by Shannon (1965) on bovine spermatozoa also showed that there was a significant interaction of storage time and dilution rate in relation to survival time (Shannon 1965). Future studies involving the storage of honey bee spermatozoa should

consist of regulation of spermatozoa concentration to standardized samples, in addition to finding the best dilution rate for stored honey bee sperm. Homogeneity of sperm cells / μL would discredit variation in sperm concentration as a factor contributing to spermatozoa death.

The results of this study clearly show that coumaphos should not be used in colonies where drones are produced. Compared to controls and other miticide treatments sperm viability of drones exposed to coumaphos was significantly lower initially and continued the trend through the 6 week sampling period. It is possible that extreme viability decreases observed in spermatozoa stored from coumaphos exposed drones could affect the performance of queens if mated with these drones. Queen performance could drastically decline 6 weeks after insemination or mating, leading to queen failure, and thus partially explain the current problems associated with maintaining productive queens in colonies.

6. Summary

Our knowledge of honey bees has advanced from the popular misconception during Aristotle's time that worker bees developed form oxen, and drones came from horses (Caron 1997). However, there are still many aspects of bee biology and behavior that we do not understand. In the United States researchers are currently trying to determine the causes of Colony Collapse Disorder (CCD). CCD is characterized by the presence of honey stores and capped brood in a hive, but a complete absence of adult bees in the colony (Henderson et al. 2007). A more deeply rooted problem that has been afflicting the beekeeping industry has to do with the continuing loss of productive queens in colonies. Beekeepers have been troubled with problems such as rapid queen supersedure following the introduction of a new queen, as well as an inability of colonies to naturally re-queen themselves (Sanford 2001). The problems associated with maintaining functional queens in hives have coincided with the increased use of miticides for parasitic mite control.

Miticide application became a mandatory practice for most beekeepers in the United States shortly after the introduction of the tracheal mite (*Acarapis woodi*) in 1984 (Shimanuki et al. 1994) and the varroa mite (*Varroa destructor*) in 1987 (Shimanuki et al. 1994, Needham 1998). Bees appear to have developed some resistance to tracheal mites, but varroa mites are still a major contributing factor to the loss of honey bee colonies (Finley et al. 1996). Since their introduction to the U.S., the impact of the mites has changed the pest management systems of beekeepers, and devastated feral and commercial honey bee populations (Shimanuki et al. 1994). The loss of colonies to tracheal and varroa mites in the U.S. has been widely reported (De Jong 1997, Wilson et al. 1997, Sammataro et al. 2000). Unfortunately, over the last 20 years varroa mite control strategies in the U.S. have centered on the use of chemical acaricides, and led to an over reliance on chemical use (Stanghellini and Raybold 2004).

Acaricide treatments expose not only mites to the chemical, but eggs, larvae, and adult honey bees as well. Several studies have shown negative effects of miticides on the health of colonies and individuals within the hive (Cox et al. 1989, Duff and Furgala 1992, Currie 1999, De Guzman et al. 1999, Rinderer et al. 1999, Westcott and Winston 1999, Ellis et al. 2001, Fell and Tignor 2001, Skinner et al. 2001, Haarmann et al. 2002,

Pettis et al 2004). The most alarming findings regarding the use of miticides have been the effects on the reproductive physiology of queens and drones.

Sokol (1996) and Currie (1999), for example, demonstrated that queens exposed to fluvalinate may lead to problems such as queen loss, supersedure, and queen mortality. Other reports (Collins et al. 2004, Pettis et al. 2004, Haarmann et al. 2002, Fell and Tignor 2001) have shown the negative effects of coumaphos on queens, including high mortality, lowered body and ovary weights, decreased sperm numbers in the spermatheca, lowered acceptance of larvae grafted for queen cells, and reduced numbers of queens functioning in colonies after 6 months. Miticide use in honey bee colonies has also been shown to reduce drone survival (Rinderer et al. 1999) and production (De Guzman et al. 1999), lead to reductions in body and mucus gland weights (Rinderer et al. 1999), and to reductions in spermatozoa production (Fell and Tignor 2001).

To date, the effect of miticides on the viability of sperm produced by drones exposed to miticides during development has not been examined. The first objective of this project, therefore, was to look at the effects that the commonly used miticides fluvalinate (Apistan®), coumaphos (Check Mite+), and Apilife VAR® (74.1% thymol, 16.0% eucalyptus oil, 3.7% L-menthol, and 6.2% other) have on sperm viability and production in exposed drones. Drones were reared in colonies treated with the recommended label dose of each miticide, and were then collected at the age of sexual maturity for examination. One seminal vesicle was removed from each drone and sperm contained within was counted using a hemacytometer. Sperm obtained for viability assays were acquired by force ejaculation of drones, followed by a rinsing technique. Dual fluorescent stains propidium iodide and Hoechst N. 33342 (H342) were used for the assessment of sperm viability.

The results of this study indicate that exposure of drones to Apilife VAR® and coumaphos significantly lowered ($P<0.0001$) sperm production. A mean sperm number of 5.99 ± 0.28 (S.E.) x 10^6 was found for the control drones whereas the treatment means for fluvalinate, thymol, and coumaphos were 5.33 ± 0.21 (S.E.) x 10^6, 4.78 ± 0.36 (S.E.) x 10^6, and 2.98 ± 0.24 (S.E.) x 10^6 spermatozoa, respectfully. Coumaphos treatments also led to significantly lower ($P<0.0001$) sperm viability in drones compared to the drones from control colonies. The mean viability of sperm varied from a low of 93.2% ± 0.7

(S.E.) in drones exposed to coumaphos, to a high of 98.5% ± 0.4 (S.E.) for drones reared in control conditions. Rearing drones in colonies treated with Apilife VAR® or fluvalinate had no significant effect on sperm viability.

The second objective of this study was to look at the effects of the same three miticides on the amount and viability of sperm contained in the spermatheca of queens exposed during development. Queens from each treatment were collected after mating and the initiation of egg laying and were dissected to remove the spermatheca. The contents of the spermatheca were assessed for total sperm number and viability. Queens reared under coumaphos treatments failed to develop, and no mated queens were obtained for analysis.

The viability of sperm from all 24 queens examined varied from 83.0% to 95.9%. The mean sperm viability varied from a low of 86.4% ± 0.8 (S.E.) in queens exposed to Apilife VAR®, to a high of 91.7% ± 0.9 (S.E.) for queens reared in control conditions. The results indicate that exposure to Apilife VAR® significantly decreased ($P<0.0016$) the viability of spermatozoa contained in the spermatheca of mated queens. Rearing queens in colonies treated with fluvalinate had no significant effect on the viability of sperm stored in the spermatheca.

The numbers of sperm contained in the spermatheca ranged from 3.65×10^6 to 6.97×10^6 spermatozoa for all queens. A mean sperm number of 6.29 ± 0.26 (S.E.) $\times 10^6$ was found for the control queens and the treatment means for fluvalinate, and Apilife VAR® were 5.35 ± 0.38 (S.E.) $\times 10^6$, and 5.14 ± 0.38 (S.E.) $\times 10^6$ spermatozoa, respectively. No significant differences were found between miticide treatments in regard to the amount of sperm contained in the spermatheca of miticide exposed or control queens.

The final objective of this research was to test the effect of miticides on the viability of stored sperm obtained from drones exposed to miticides during development and sexual maturation. Drones were reared in hives treated with one of three miticides (fluvalinate, coumaphos, and Apilife VAR® (thymol)) and collected after maturation. Semen was collected from multiple drones from each colony and pooled. The pooled samples were subdivided and held in capillary tubes for up to 6 weeks. Random samples

were taken from each treatment (n = 6 pools) once a week for 6 weeks and analyzed for viability.

The viability of stored sperm from coumaphos treated drones was the lowest with an initial mean viability of 86.2% ± 0.5 (S.E.). Sperm viability remained significantly lower throughout the 6 week sampling period, but had a sharp decline between week 5 and 6, reaching the lowest value at the end of week 6 (48.6% ± 2.8 (S.E.)). Exposure of drones to coumaphos during development significantly reduced sperm viability compared to other treatments and controls for all weeks (0-6). Significant differences were also observed between weeks 5 and 6 for all treatments and for viability of the controls from the initial viability to week one ($P < 0.0152$).

In conclusion, this project has supplied relevant information involving the effects of miticides on the reproductive physiology of queen and drone honey bees. The use of Apilife VAR®, and coumaphos should be limited in colonies, particularly in those colonies used for queen and drone production. This study has also shown that the use of miticides negatively affects sperm production and viability in drones, in addition to lowering the viability of sperm contained in the spermatheca of queens. Finally, the *in vitro* study involving sperm storage indicates that latent effects from drone exposure to miticides during development can affect sperm viability over time. The significant decline in sperm viability observed after 6 weeks could also occur in the spermatheca of queens, and could explain some of the current problems associated with queen failure and supersedure.

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8. Vita

Lisa Marie Burley was born on May 4, 1981 in Lansing, Michigan. She is the daughter of Warren and Diane Burley and has a brother, Michael. Lisa attended The University of Michigan, from where is earned a Bachelor of Science Degree in Ecology and Resource Management in April 2003. Post graduation she was granted a resource management internship working for the Virginia Department of Conservation and Recreation. She moved to Blacksburg, VA to pursue a Master's degree in Entomology at Virginia Polytechnic Institute and State University in the summer of 2004. She was an active member of the W.B. Alwood Society, serving as the secretary in 2005, and a tour coordinator in 2006. In the spring of 2007 she received the James McD. Grayson Scholarship for outstanding achievement in graduate study from the Department of Entomology at Virginia Tech. While working toward her master's degree Lisa was very active in extension, participating in several beekeeping workshops, and giving numerous talks to beekeeping groups throughout the state of Virginia. In August 2007 she was invited to present her master's research at the Eastern Apicultural Society in Newark, Delaware. She received her Master of Life Sciences in Life Sciences Summer of 2007.