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II: Instrumentation insemination

PI: Propidium iodide

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Preface

Chapter I titled “Preservation of Honey Bee (*Apis mellifera* L.) Semen” is a review of the pertinent research literature relating to this master’s project. Chapter II has been written as a scientific article and for submission to the scientific periodical, *The Canadian Entomologist*. The first author of both the review and the article is the master’s degree candidate, Marilène Paillard. The co-authors are Andrée Rousseau M.Sc., research professional at the Centre de recherche en sciences animales de Deschambault, Janice L. Bailey Ph.D., professor at the Animal Sciences Department and vice Dean of research at the faculté des sciences de l’agriculture et de l’alimentation of l’Université Laval, and Pierre Giovenazzo Ph.D., professor at the Biology Department of Laval University. These persons contributed intellectually and technically to the project and corrections and participated in writing the article.

Introduction

Honey bees (*Apis mellifera L.*) are critical players in the agricultural industry for food production as they account for the vast majority of insect pollinators. At least 35% of the human diet depends on these pollinators (Klein et al. 2007). An average of 1/3 of food on our plate is the result of insect pollination, mostly honey bees. Food and fibres from insect pollination was reported to be valued at approximately CAD $2 Million/year in Canada (Darrach and Page 2016) and CAD $217 Billion/year globally (Gallai et al. 2009).

Furthermore, honey bees are the source of other industries based on the production of honey, royal jelly, wax, pollen, and propolis. Honey brings the highest income for beekeepers, while the other products only represent 6% of honey bee revenues (Massicotte 2015). In 2014, there were approximately 8 777 beekeepers registered in Canada, owning 694 217 hives, and 309 beekeepers in the province of Québec (49 635 hives) (Massicotte 2015; Statistique Canada 2015). Hives, also known as colonies, are not equally distributed between beekeepers. Many beekeepers have 1-5 hives, while others possess more than a hundred, even thousands of colonies. An average-sized colony produces about 43 kg of honey annually (Massicotte 2015). In Québec, honey production was about 1,926 metric tons in 2014, which represented revenues of $13.4M (Massicotte 2015). In Canada, honey production is about 36,993 metric tons and worth $201.3M (Statistique Canada 2015). Furthermore, many beekeepers rent their hives for crop pollination at a cost of about $115.5 per colony for the cultivators (Massicotte 2015). In 2014, 44,214 colonies were rented in the province of Québec, resulting in an income of $5.1M to beekeepers.

In the last decade, however, there have been important losses of honey bee colonies worldwide, including Canada (Moritz and Erler 2016). These population declines have been attributed to various factors. The monocultures depending on honey bee pollination are highly prominent in the province of Québec, Canada, which greatly limits food diversity to the honey bees (Girard et al. 2012). Moreover, the lack of food diversity combined with high unintentional pesticide exposures make the honey bees more vulnerable to parasites (Goulson et al. 2015). The most devastating parasite of the honey bee health is *Varroa destructor*, a parasite mite that feeds on honey bee larvae (Allen Wardell et al. 1998; Bromenshenk et al. 2010; Currie et al. 2010; Neumann and Carreck 2010; Ratnieks and Carreck 2010). The combination of these factors (pathogens, food restrictions of homogenous crops, moving stress of hives from one crop plant to another, and pesticide exposure) increases the risk of losses of honey bees (Vanengelsdorp et al. 2009; Goulson et al. 2015). Adding to their vulnerability, many of the honey bee subspecies have been replaced by commercially available stock, which has further reduced the genetic diversity of *Apis mellifera* (Buchler et al. 2014).

Preservation of honey bee sperm is an effective strategy to protect genetic diversity and to facilitate genetic lineage to prevent as much colony loss as possible. The goal of this review is to summarise honey bee reproduction and provide an overview of drone semen preservation research and results since the early 1960s.

Chapter I: State of Knowledge

1.1 Honey bees

The honey bee, *Apis mellifera* Linnaeus (Hymenoptera: Apidae) originates from Europe, Africa and the Middle East, and is now widespread around the world (Seeley 1995). Honey bees have survived in various climatic conditions and evolved into many different subspecies (Louveaux et al. 1966). Honey bees are social insects of the order Hymenoptera, which includes more than 100,000 species of sawflies, wasps, ants and bees (Honeybee Genome Sequencing 2006). Social insects live in a colony where parents collaborate with their offspring, who give up their own reproduction for the reproductive success of their parents.

There are three castes in a honey bee colony (Figure 1.1): the queen, the female workers, and the drones (males). The queen honey bee, like other social hymenopterans, is the only fertile female and is thus responsible for maintaining the colony's population and productivity. Her offspring are mainly sterile female workers, which are the most abundant caste in the colony. Workers are responsible for most of the tasks in the hive, such as protecting the colony, cleaning the hive, collecting pollen and nectar from flowers to produce honey, nursing the larvae, and constructing the wax combs. Depending on the worker's age, she will have different tasks in the hive (Lindauer 1952). For example, the youngest workers will nurse the larvae while the oldest collect nectar for the colony. The principal function of the drones is to mate with a virgin queen (Gullan 2010; Winston 2010). Depending on the time of year, the colony may contain approximately 50 to 60 thousand workers and a few thousand males (Winston 2010; Koeniger et al. 2014).

Figure 1.1: Honey bee caste differentiation: Left, the larger queen, with the red spot on the thorax, surrounded by smaller workers; Right, the drone (Photo credit: Marilène Paillard).

1.2 Caste Development

Honey bees are holometabolous insects, meaning that individuals go through multiple phases during development, including a complete metamorphosis, before emerging as an adult. Four stages occur within the same comb (Figure 1.2): egg, larva, pupa and adult (Jay 1963). The embryo develops during the egg stage. Afterwards, the larva emerges from the egg and grows rapidly. During the pupal stage, the organism is in an inactive state, and metamorphosis occurs. Finally, the adult honey bee, either worker, queen, or drone, emerges from the comb and immediately initiates its functions (Winston 2010).

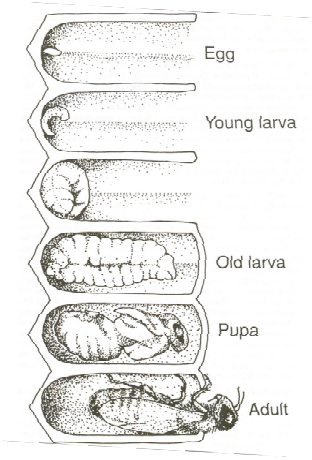

Figure 1.2: Representation of the four developmental stages of the honey bee: egg, larva, pupa and adult (Winston 2010).

The duration of development before emerging into an adult differs for each caste. Queens, workers and drones take 16, 21 and 24 days to develop, respectively (Winston 2010). Female caste differentiation depends on the quality and quantity of food provided by the worker bees during their early development (Haydak 1943; Jay 1964). Female larvae can develop either into queens or workers during the first three days after the egg has been laid (Shuel and Ddxon 1960; Jay 1964). The first 48 h after egg emergence, the female larva is bipotent, meaning that she can follow one of two different developmental pathways (Winston 2010). During these two days, female larvae are fed royal jelly and it is at the third day that feeding differs and determines which developmental path is executed. Royal jelly is a creamy and viscous glandular secretion from the worker's hypopharyngeal gland (Hebert 2010), composed of water (60-70%), sugar (10-16%), crude protein (12-15%), lipids (3-6%), traces of salts, free amino acids, and vitamins (reviewed in Buttstedt et al. 2013). Of note, royal jelly contains a protein called royalactin that helps larvae develop more ovarioles in the ovaries (Kamakura 2011). The queen larva receives high amounts of nutrient-rich royal jelly throughout all the development stages, ensuring her reproductive capacity. In contrast, workers receive less royal jelly than future queens and feeding will be switched to honey and pollen after two days of development (Weaver 1955,1957). Workers'

reproductive organs, therefore, remain undeveloped (Page and Peng 2001). This nutritionally-induced developmental pathway is driven by epigenetic changes via DNA methylation (Kucharski et al. 2008)

Like other hymenopteran insects, honey bees are haplodiploid. Females (workers and queens) developed from fertilized eggs (diploid), and drones from unfertilized eggs (haploid) (Beye et al. 2003). Thus the male offspring has 50% of the maternal genome. The sex-determination mechanism in hymenopteran insects is different from most animal species, including many insect orders, because they lack sex chromosomes (Beye et al. 2003). There is no sex-specific chromosome as in mammals, for example, where males have the XY chromosomes and females have the XX chromosomes. The haploid honey bee drones randomly receive half of the queen's genome. It is, therefore, the quantity of one specific locus, the "complementary sex determiner" (*csd*) that determines the offspring sex (Mackensen 1951; Beye et al. 2003; Honeybee Genome Sequencing 2006). Eggs containing one *csd* locus become males, and eggs with a diploid locus become females (workers or queens). Furthermore, the fertilized egg must have two different *csd* loci, originating from unrelated parents, to create a female worker; otherwise the egg will develop as a diploid male (Mackensen 1951). When incestuous mating occurs, workers will usually cannibalize the diploid male larvae (Woyke 1965).

1.3 Sexual Maturation

1.3.1 Queens

Queens develop for 16 days before emerging as an adult: three days as an egg, six days as a feeding larva and seven days as a pupa (Winston 1987). She becomes sexually mature and takes her mating flight 5-6 days after emergence (Ruttner 1956). Three to seven days after mating, oogenesis begins in the queen's

two ovaries (Kaftanoglu and Peng 1982). Each queen ovary contains approximately 150 ± 180 ovarioles, contrarily to only 2-12 ovarioles in workers (Page and Peng 2001). Ovarioles are long tubes, located in the ovaries of most insects, that produce eggs (Snodrass 2010). The queen can lay up to 2 000 eggs in a single day, or approximately 200 000 eggs in one year (Winston et al. 1991).Egg production tends to decrease as the queen ages. She usually will live up to 3 years (Seeley 1978; Page and Peng 2001), during which time she will lay up to 1.0 million fertilized eggs throughout her life (Baer 2005).

Competition occurs between young queens, coming from the same hive. When they are newly emerged, sister queens fight each other until there is only one survivor. Queens use their mandibles and stinger as weapons to eliminate the opponent. The defeated queen, stung with venom, falls paralyzed and dies. The surviving queen then searches the entire hive to eliminate additional competitor sisters (Butz and Dietz 1994; Pflugfelder and Koeniger 2003). Young queens also attack developing queens who have not yet emerged from cells. The last queen remaining inherits the hive. Meanwhile, the old queen, who is the mother of the young queen, will leave the hive with half the workers to establish a new colony. This form of colony reproduction is called colony fission (swarming) (Winston et al. 1991).

The young queen remains in the hive for few days to eat and mature, following which the behaviour of workers changes and becomes more aggressive toward the queen. They bite her, presumably to “encourage” her to take her mating flight (Koeniger et al. 2014). It is important for the honey bee colony that the new queen’s development is as fast as possible. When the old queen leaves the hive with half of the colony or dies, there is a time lapse where no brood is produced. Therefore, the presence of newly mated queen must take the least possible time to repopulate and prevent colony collapse (Koeniger et al. 2014).

1.3.2 Spermatheca

The spermatheca is a female organ that stores sperm from previous copulations (Figure 1.3a). Although spermathecae are present in most insects, the honey bee queen is exceptional in being able to store semen for years while maintaining sperm viability (Verma 1973; Klowden 2013).

The mechanism by which the sperm remain fertile for such a long period in the spermatheca remains to be elucidated, however, a high protein concentration (8.5-15.3 mg/ml) is present in the spermathecal gland secretions (Klenk et al. 2004; Wegener et al. 2013) and may play a role on long-term storage of sperm (Klenk et al. 2004).Some proteins are thought to be antimicrobial in the spermatheca (Collins et al. 2006). Other proteins related to sperm energy metabolism are found in both the ejaculated semen and the stored semen but in higher concentrations in ejaculated semen (Poland et al. 2011).

Since the environment of the queen's spermatheca is aerobic and sperm maintain their respiratory activity during storage, sperm are at risk of oxidative damage (Weirich et al. 2002; Collins et al. 2004). Therefore, antioxidants such as catalase, glutathione S-transferase and superoxide dismutase are found in the queen's spermatheca (Weirich et al. 2002). These enzymes protect the sperm against oxidative stress by reducing the levels of reactive oxygen species (ROS) and improve sperm longevity (Weirich et al. 2002). The spermatheca also has a high concentration of Na^+ and K^+ and a high pH (8.6) that encourage a low rate of sperm metabolism (Verma 1973).Sperm are stored in an immobile state, which also minimizes their metabolic activity. Sperm motility is then activated upon contact with the spermathecal gland secretions, when the sperm are released for fertilization (Flanders 1939; Lensky and Schindler 1967).

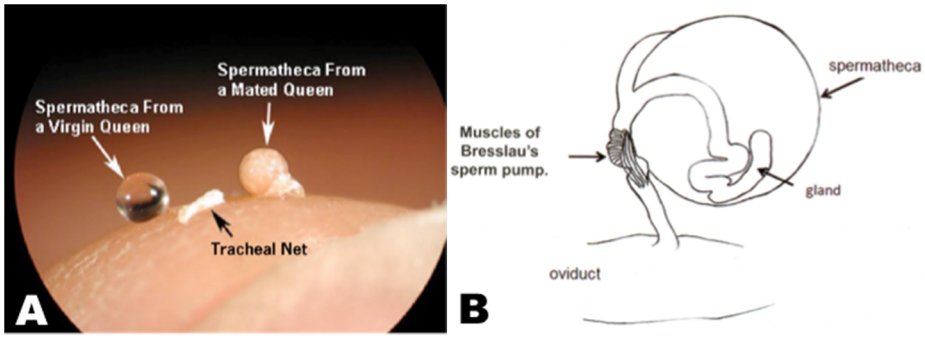

Figure 1.3: A) Spermathecae from two different queens: Left, from a virgin queen without sperm and right from a mated queen (Cobey et al. 2013). The presence of sperm is usually apparent in that from the mated queen. B) Illustration of spermathecal anatomy (Koeniger et al. 2014).

In the honey bee queen, the spermatheca is a small sphere with a diameter of 0.9-1.3 mm with a volume of about 0.45-1.15 microliters (Verma 1974). The spermatheca is located at the rear end of the abdomen above the queen's oviducts (Figure 1.3b) (Koeniger et al. 2014). It has two glands and is surrounded by a dense tracheal net that helps transport oxygen to the sperm inside the spermatheca (Koeniger et al. 2014). A narrow duct connects the spermatheca to the common oviduct (Bresslau 1905) and contains the Bresslau's sperm pump, which allows the queen to control sperm release as the egg transits the oviduct (Harbo 1979c; Koeniger et al. 2014).

The queen is able to control fertilization of the egg by measuring the brood cell with her forelegs (Koeniger 1970a,b). If the queen lays an egg in a male cell, which has a bigger diameter than an average cell, she will not release sperm to fertilize the egg. In contrast, for the female castes, the queen releases sperm to fertilize the egg before laying it in a worker or a queen cell (Koeniger 1970a,b). The queen deposits the egg on the bottom of the cell, with or without sperm.

1.3.3 Drones

A drone requires 24 days of development from egg to emergence (Koeniger et al. 2014). The drone egg hatches after three days, the time required for embryo development. The larva then feeds and grows for days. Finally, the pupal stage last 15 days (Page and Peng 2001). Drones need to be nursed by workers, during their larval stage and as young adults. The young drones stay in the warm brood area for the first days where workers feed them. Drones then move to the honeycomb regions and gradually start feeding autonomously with the food present within the colony (Winston 1987; Koeniger et al. 2014). Drones can weigh up to 196 ± 225 mg when they emerge as adults, which is the average size of a queen, but twice the size of a worker bee (Winston 1987). Before they become mature, young drones aged from five to eight days, perform orientation flights to locate the Drone Congregation Area (DCA), where they will attempt to mate with a virgin queen (Koeniger et al. 2005b). The longevity of a drone is between 20 and 40 days (Ruttner 1966; Page and Peng 2001; Rueppell et al. 2005) with an average of 21.1 days (Fukuda and Ohtani 1977).

In Eastern Canada, drone-producing season is from mid-April to late August with a peak occurring during the swarming season, which is from June to July (Rousseau et al. 2015). This period is associated with abundant food sources that are essential to produce drone broods (Lee and Winston 1987; Rowland and McLellan 1987; McNally and Schneider 1994). Since sexual maturation of drones is longer compared to queens (36 days vs. 22 days, respectively), drones must be produced and raised earlier than queens to be sexually synchronized with queens (Koeniger et al. 2014). Several factors have been linked to drone production in honey bee colonies (Conner 2008): increasing photoperiod, nutritional conditions and queen age. At the end of the summer, the queen stops laying eggs in drone cells. When drone cells are not used for brood production, they are filled with honey.

1.3.4 Sexual Maturation of Drones

Honey bees appear to be the only insect in which spermatogenesis occurs only during their developmental stages, therefore, drones have a predetermined quantity of sperm after embryo development (Bishop 1920; Page and Peng 2001). Other insects produce spermatozoa throughout their life (Bishop 1920). Formation of the male reproductive system starts early during development. Testes are formed at the embryonic stage, before the larva emerges from the egg (Figure 1.4). Spermatogonia undergo multiple mitoses, resulting in primary spermatocytes (Hoage and Kessel 1968). Spermatogenesis is initiated on the third day of the larval stage. The primary spermatocytes undergo a reductional meiosis where two cells are produced: one secondary spermatocyte containing 16 chromosomes (haploid) and one cell containing only cytoplasm and an empty nucleus. The secondary spermatocytes then undergo a non-reductional meiosis, resulting in two spherical spermatids. During the larval stage, drones grow quickly, including the testes and spermatid proliferation, due to the high food provisions from the workers (Hrassnigg and Crailsheim 2005). Spermatid multiplication stops prior to pupation (Hoage and Kessel 1968), and spermiogenesis is initiated, which is the morphological differentiation resulting spermatozoa.

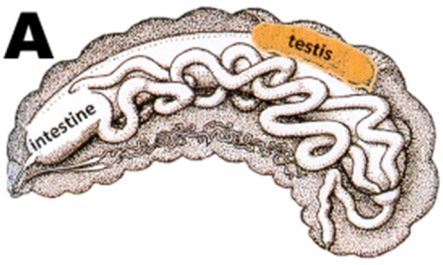

Figure 1.4: Development of testes during embryogenesis (Koeniger et al. 2014).

Approximately 2-3 days after pupal emergence, sperm start migrating from the testes to the seminal vesicles, which is the enlarged area of the vas deferens used for sperm storage, where they remain for approximately 13 days (Koeniger et al.

2014). Testes size is greatly reduced after sperm migration. Once in the seminal vesicles, the spermatozoa attach via the head to the single layer glandular cells that cover the vesicle walls. Here, it is thought that the spermatozoa go through a maturation phase where they absorb nutrients from the gland cells to become fully functional and remain so until copulation (Ruttner 1976; Koeniger et al. 2014). Little information regarding this apparent maturation is present in the literature. Additional research is required to understand bee sperm physiology during transit in the male tract.

A drone is not yet sexually mature at emergence, despite having produced mature sperm. The copulatory organ, the endophallus, is fully developed and tightly stored in the abdomen (Koeniger et al. 2014). Endophallus eversion is possible on a newly emerged drone, but no mucus and semen are present for emission because the accessory gland cells are not yet active. At 5-6 days after emergence, drones may ejaculate mucus during eversion but without semen (Ruttner 1976). Drones are sexually mature twelve days port-emergence, which is six more days required for sexual maturation of the queen (Koeniger et al. 2014). At this age, drones can completely evert their endophallus and creamy coloured semen, containing spermatozoa, can be located at the posterior extremity of the ejaculate, on top of the white mucus, which is void of spermatozoa (Figure 1.5). The fluids do not mix together.

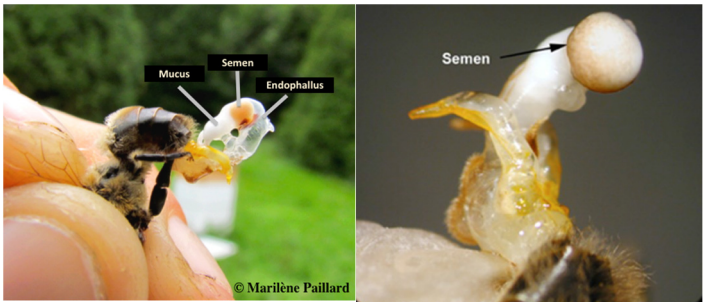

Figure 1.5: Full eversion of the male's endophallus, exposing the semen and the mucus (photo credit: Left Marilène Paillard; Right (Cobey et al. 2013)).

Drones have three accessory sex glands: the mucus gland, the bulbus gland (Woyke and Ruttner 1958), and the cornual glands. At the end of the larval stage, the mucus gland develops from the vas deferens, the duct connecting the testes to the seminal vesicles, and produces white mucus (Koeniger et al. 2014). The cornual and bulbus glands both consist of single layers of cells (Moors and Billen 2009) and are found near the extremity of the endophallus, also known as the bulb (Koeniger et al. 1996; Moors et al. 2012). The cornual glands secrete an orange-coloured secretion that helps strengthen the attachment of the mating sign in the queen's reproductive tract, while the endophallus, engorged with mucus, reinforces the connection between the drone and the queen during mating (Koeniger and Koeniger 2000; Colonello and Hartfelder 2005). The drone's reproductive secretions are predominantly composed of proteins, except from the cornual glands, which produce lipids (Colonello and Hartfelder 2003; Moors and Billen 2009). The mucus glands start secreting mucus after the drone's emergence and are filled at five to six days of age (Ruttner 1976). Each mucus gland contains approximately 50-60 µg of protein (da Cruz Landim and Dallacqua 2005). The mucus is used to produce a mating sign in the queen after a successful copulation (Koeniger 1988). The drone's bulb is filled with mucus and other secretions (Figure 1.6). The chitin plates ensure that no secretory fluid will penetrate inside the newly mated queen's reproductive tract, except for the semen, containing sperm and the seminal fluid, which is described later. The mating sign represents 10% of the drone's body weight (15 mg) (Koeniger et al. 2014).

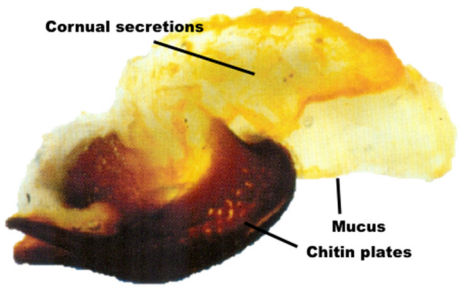

Figure 1.6: The mating sign retrieved from the sting chamber of a newly mated queen (Koeniger et al. 2014). The bulb, with the chitin plates, originating from the endophallus, retains secretory fluids such as the mucus and the cornual secretion from entering the queen's lateral oviducts.

1.3.5 Drone Semen

Semen is the combination of sperm and seminal fluid produced by the seminal vesicles. The seminal fluid is mostly composed of more than a hundred proteins that are different from the other gland secretions. Seminal fluid proteins have many functions, such as defense against microbial attacks or oxidative stress, energy production for sperm, and metabolism of carbohydrates and lipids (Baer et al. 2012; Gorshkov et al. 2015). The concentration of proteins involved in signalling and maintaining viability differs by 16% among genetic lineages, which could influence sperm competition by reducing fertilization success of other drone sperm (Baer et al. 2012). The seminal fluid also contains sugars such as fructose, glucose, and trehalose, which serve as energy sources for the spermatozoa (Blum et al. 1962; Verma 1974).

Drones produce an average of 1.5-1.7 microliter semen with approximately 7.5 million spermatozoa/µl (Rousseau et al. 2015). Sexually mature drones have a yellowish creamy coloured semen. The semen becomes darker, thicker and the semen volume decreases with age (Woyke and Jasinski 1978; Czekonska et al. 2013; Rousseau et al. 2015). To the best of our knowledge, the reason is still unknown. Insemination with thicker semen has a risk of blocking the queen's oviducts and increases the likelihood the queen will die (Woyke and Jasinski 1978). Contradictory reports have demonstrated that sperm viability decreases with age (Locke and Peng 1993), while others observed an increased viability with age (Czekonska et al. 2013). Rousseau (2015) reported that age has no effect on sperm motility and viability. It is still unclear whether sperm viability increases or decreases with age, however, the drone's ability to copulate varies with age, depending on the colony strength and quality (Rhodes et al. 2011).

1.3.6 Spermatozoa

Most insects have very long spermatozoa with an elongated head. Honey bee spermatozoa measure 250-270 µm in length (Lino-Neto et al. 2000) with the head measuring 8 to 10 µm (Figure 1.7) (Peng et al. 1993). The whole cell is 0.7 µm wide (Peng et al. 1993). The head, containing the nucleus filled with DNA, is asymmetric and is composed of a bi- or tri-layered acrosomal complex (Lensky et al. 1979; Peng et al. 1993). The length of the acrosome is approximately 5 µm (Lensky et al. 1979). Unlike vertebrates, honey bee sperm lack the middle piece (Rothschild 1955). The tail is a flagellum composed of two mitochondrial derivatives, an axoneme, and two triangular shaped accessory bodies (Peng et al. 1993; Lino-Neto et al. 2000). The axoneme consists of nine single accessory microtubules, nine doublets, and a central singlet pair (9 + 9 + 2). Two mitochondrial derivatives are parallel to the axoneme, starting at the base of the nucleus and finishing at the end of the flagella (Rothschild 1955). The necessity for sperm capacitation and the acrosome reaction during the fertilization process are still unknown in the insect taxa compared to many other animal species (Peng et al. 1993).

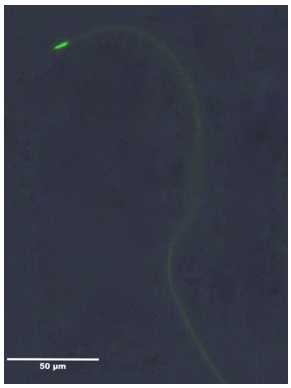

Figure 1.7: Live spermatozoa of the honey bee drone coloured in green florescence with SYBR-14, observed in a fluorescent microscope at 400X (photo credit: Marilène Paillard). The SYBR-14 penetrates the cell membrane and binds to DNA.

1.4 Mating

Honey bees are polyandrous and this behaviour is not found in most social insects (Gencer et al. 2011). Polyandry means that the queen mates with multiple males (Koeniger and Koeniger 2000; Palmer and Oldroyd 2000). It is an important reproductive phenomenon for honey bees because queens mated with only one drone produce weaker colonies (Fuchs and Schade 1994; Moritz and Fuchs 1998; Tarpy 2003; Page et al. 2006). Consequently, workers' genetic profiles are more diversified and optimise their productivity (Koeniger et al. 2014). Honey bees have an intense male-biased sex ratio estimated at 1,000 to 2,000 males per reproductive female (Page and Metcalf 1984; Koeniger et al. 2014). The sex ratio is usually 1:1 in most animal species, including insects of the order Hymenoptera such as wasps, solitary bees and many ant species (Koeniger et al. 2014).

Honey bee mating takes place in the afternoon, during flight, when weather conditions are optimal, which must be suitable for both drones and the queen to fly to DCAs (Ruttner 1956; Koeniger et al. 2014). Weather is an important mating factor in northern regions. The ideal temperature for the mating flight is 18˚C for drones and 20˚C for the queen (Koeniger et al. 2014). Furthermore, mating will only occur during sunny days to avoid strong winds and rain (Koeniger et al. 2014). It is not known how drones and young queens, residing in the hive, are able to sense these weather conditions, since the hive is thermo regulated by workers.

Drones leave their hive around 2 pm until 5 pm (Koeniger et al. 2014). Both drones and queens use the light cycle to regulate their flight (Koeniger et al. 2014). They can take several mating flights lasting up to 25 minutes each until copulation occurs (Witherel 1971; Koeniger et al. 2014). Drones can execute a maximum of about six mating flights daily (Koeniger et al. 2014). As for the queen, approximately one week after emerging, she executes short orientation flights of about five minutes (Koeniger et al. 2014). The queen then flies to a DCA about 30

to 60 minutes after drones have left the hive. Her mating flights can last between 13 (Taber 1954) and 21 minutes (Woyke 1960), and occur between 2:30 and 4 pm. The DCA is already established when the virgin queen arrives to mate (Winston et al. 1991). The young queen mates for a short period of time (2-3 days) and, once she starts laying eggs, never mates again (Winston 1987). She can have one or multiple mating flights, sometimes up to three flights, depending on the volume of semen deposited (Taber 1954; Ruttner 1980; Tarpy and Page 2001).

Honey bee queens and drones originating from the same hive have adapted behaviour to avoid inbreeding by flying at different DCAs. Drones fly to closer DCAs, while the young queen will go to a further DCA (Koeniger et al. 2014). The energy and the flight time are very valuable to drones since they have to stay long periods of time at DCAs to locate and possibly mate with the queen. Therefore, drones fly at closer DCAs to preserve their time and energy. Queens can focus their energy on flying further, since mating with multiple males only takes few minutes (Koeniger et al. 2014).

The virgin queen heads to a DCA where more than 10,000 to 30,000 drones of different genetic lineages, originating from approximately 240 colonies, await (Gary 1963; Koeniger 1986; Loper et al. 1987; Baudry et al. 1998). During the mating flight, drones fly in the DCA at 15 to 60 m above the ground and form a circle of 60-200 m diameter (Gary 1962,1963; Loper et al. 1987). Drones are attracted to the queen's sex pheromones, of which the main component is 9-ODA (9-oxo-decenoic acid). They are also attracted visually, as she arrives at the DCA (Koeniger and Koeniger 2000). Once in the DCA, over 20-100 drones pursue the queen, forming a "mating comet" behind her, ready to copulate (Koeniger 1990). These drones follow her and, eventually, one will grasp the queen and mate.

The copulation between a queen and a drone takes a few seconds (Gary 1963). The flying drone mounts the queen from behind (Figure 1.8a) (Koeniger et al.

2014). The male holds the queen's abdomen from each side with his front and middle legs. Once the male holds the queen tightly, the drone orients the tip of his abdomen in the queen's open chamber and inserts the first half of his endophallus (Koeniger 1986; Woyke 2008). Since the endophallus is folded in the drone's abdomen, muscles of the abdomen contract simultaneously. A large amount of hemolymph then enters the endophallus from the abdomen (Figure 1.8b) (Koeniger et al. 2014).

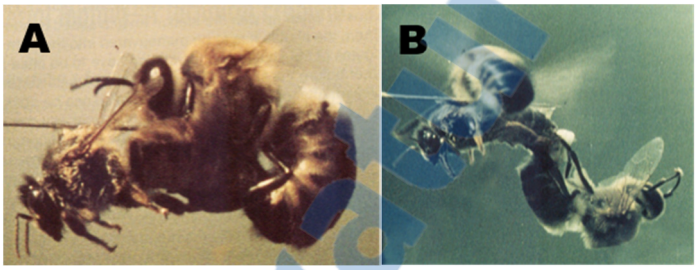

Figure 1.8: A) Drone mounting the queen from behind, ready for copulation (Koeniger et al. 2014). B) The drone is falling backwards, paralysed, after the first eversion of the endophallus (Koeniger et al. 2014).

When half of the eversion is complete, the drone's wings stop moving and its body is paralysed since almost all of the hemolymph is pressed into the endophallus (Koeniger 1986). The male swings backwards, still attached to the queen by his legs (Koeniger et al. 1979), and the queen contracts her sting chamber to complete the full eversion of the drone's endophallus. Sperm are then transferred from the endophallus to the queen's oviducts where the vaginal valve and sphincter hold back the sperm from exiting the reproductive tract (Koeniger et al. 2014). Sperm-free white mucus, found at the extremity of the endophallus, pushes the semen to migrate from the endophallus to the queen's lateral oviducts (Woyke 2010). Sperm transfer occurs in a sterile environment, because the drone's membranous endophallus seals the queen's vagina, thus no communication between the exterior environment and the queen's oviducts occurs (Koeniger et al. 2014).

Once ejaculation completed, the paralysed drone dies. After the mating, the male leaves the detached bulb from the endophallus as a “mating sign” in the queen’s sting chamber (Woyke 2010). The mating sign left by the drone increases the queen’s visibility in the DCA to attract additional drones (Koeniger 1990; Koeniger 1991). The following drone removes the previous mating sign to copulate with the queen (Koeniger 1984).This male attracting system reduces the duration of the queen’s mating flight.

The queen copulates successively (Gries and Koeniger 1996), with an average of 17 drones during one or more mating flight (Adams et al. 1977). After this mating period, the queen never copulates again. The queen then returns to the hive and the workers remove the mating sign of the last copulation (Koeniger et al. 2014). The queen receives approximately 200 million spermatozoa in her lateral oviducts. However, the spermatheca can only retain 5-7 million sperm (Woyke 1962; Koeniger 1991). Therefore, the sperm of some drones will not successfully reach the spermatheca. The excess will be expelled out of the queen’s oviducts, by contracting abdominal muscles, as the retained sperm migrate into the spermatheca. Sperm migration is complete approximately 40 h after mating (Woyke 1983).

Many mechanisms have been proposed to explain the migration of the sperm from the queen’s lateral oviducts into the spermatheca: oviduct contractions (Ruttner and Koeniger 1971), spermatozoa motility (Collins 2000a), the spermathecal pump (Koeniger et al. 2011), and even flagellar movement of sperm (Tofilski 2014). The queen uses the stored sperm for fertilization over her lifetime (1-3 years).

1.5 Drone Competition

The competition between males happens individually, meaning that they do not “fight” physically against each other to win the queen. It is the fittest males who can overcome obstacles and survive to copulate with the queen. Drones must be able to locate the DCA and the queen faster than other drones to copulate. Therefore, queens potentially mate with drones that have a better flight ability (Jaffe and Moritz 2010). The chance of any one drone mating with a queen is estimated to be 0.0001% (Koeniger et al. 2014).

1.6 Sperm Competition

It is still unclear whether sperm competition occurs in honey bees and how it works (Moritz 1986; Harbo 1990; Woyciechowski and Krol 1996; Shafir et al. 2009). Sperm competition may occur after mating, within the oviducts, during sperm storage in the spermatheca or at the time of fertilization (Woyciechowski and Krol 1996; Shafir et al. 2009; Tofilski et al. 2012). Some proteins found in the seminal fluid might displace other males’ sperm (Harshman and Prout 1994), owever, it has not yet been demonstrated in honey bees(Woyciechowski and Krol 1996). Usually, spermatozoa from the same male will not compete against each other (Parker and Pizzari 2010). Studies have demonstrated that sperm viability is reduced when mixed in seminal fluids of other males than their own seminal fluid (den Boer et al. 2010). However, these results are contradicted by another study, which found that sperm viability is not affected, and drones may possibly have sperm polymorphism (Tofilski et al. 2012).

Because of the extreme male-male competition and the high requirements of sperm by the polyandrous queen, males have required a high level of sperm viability (Hunter and Birkhead 2002). Moreover, since the queen mates for only a short period, natural selection for superior semen, which is considered as a large

ejaculate filled with highly viable sperm, is important (Hunter and Birkhead 2002; Simmons 2002; den Boer et al. 2008). To perpetuate their genetic lineage, drones should maximize the number of spermatozoa produced. The number of sperm can vary between 3 and 12 million, depending on the drone size (Berg et al. 1997; Schluns et al. 2003; Koeniger et al. 2005a) and genetic lineage (Rousseau et al. 2015).

Sperm viability plays a crucial role during fertilization, and, therefore, sperm competition (Franck et al. 2002), which is more likely to occur at this time because the queen releases 4 to 25 sperm (Harbo 1979c; Yu and Omholt 1999) from the spermatheca (Harbo 1990). Drones producing more viable motile sperm increase their chance to fertilize eggs. Furthermore, the length of the sperm can vary from 250 to 270 µm (Lino-Neto et al. 2000). Theoretically, the longest sperm will reach the spermatheca fastest, therefore, increasing their chances of fertilizing the egg (Gomendio and Roldan 1991; Montgomerie and Briskie 1992; Gage and Gage 1994; Morrow and Gage 2000). Studies on bumble bees (*Bombus terrestris*), demonstrated that sperm length is a genetic trait that responds to selection (Koeniger et al. 2014). In polyandrous bumble bee species where queens have multiple mating, drones produce longer sperm than in monogamous bumble bee species (Brown et al. 2002; Baer et al. 2003).

Spermatozoa can potentially cooperate together by producing a pseudopodium, a foot-like projection, to more rapidly reach the spermatheca (Tofilski 2014). Sperm coordinate their flagellar beats so they can swim forward and increases their chance of fertilizing more eggs (Tofilski 2014). To our knowledge, it is unknown if the sperm cooperation happens between sperm coming from the same male, or not.

1.7 Instrumental Insemination

Instrumental insemination (I.I.) is performed in many species. Instrumental insemination is a crucial technique to control animal breeding to accelerate genetic selection. I.I. allows mating of domesticated honey bees to bypass the DCA. Many factors such as unfavourable climate and undesirable drones from other colonies can negatively affect genetic improvement through natural breeding selection. In 1927, I.I. was first successfully reported in honey bees (Watson 1927). The technique was then perfected during the 1940's - 1950's (reviewed in Cobey 1983; Laidlaw 1987). Instrumental insemination is currently a highly successful method for genetic control, for research, and for stock improvement (Cobey 2007), even though this technique is difficult to execute.

Instrumental insemination is difficult to introduce in the commercial industry because of the technical complexity and is thus more often used during scientific research. Therefore, it requires a minimum of expertise to perform I.I., such as advanced knowledge breeding principles and beekeeping skills to produce queens and drones (Cobey et al. 2013).

The success of I.I. depends on adequate semen quality (Collins 2000a). Males must be between 2 and 3 weeks of age, because sperm viability decreases with age and fewer sperm migrate to the spermatheca. Moreover, as mentioned earlier, semen of older males will leave residues in the queen's lateral oviducts, thus increasing the risk of queen mortality (Woyke and Jasinski 1978). The minimum sperm viability required for queen I.I. was calculated to be 43% (Collins 2000a,2004). If sperm viability is lower, the queen will lay more drones (unfertilized eggs) and fewer workers (fertilized eggs). Therefore, the colony will die because of the lack of workers.

1.8 Semen Preservation

The development of I.I. in honey bees, has stimulated interest in semen preservation. The use of stored sperm greatly facilitates I.I., genetic improvement of the colony, and prolonging the honey bee production season. Both short and long-term semen conservation enhances selection and genetic improvement in honey bee populations (Collins 2000b). Thus, it would help developing superior honey bee stocks that are resistant to certain parasites such as varroa mites (*Varroa destructor*), for example (Collins 2000a). Furthermore, shipping honey bee semen instead of live drones would reduce the risk of spreading bee pathogens (Cobey 2007).

Additionally, in northern climates, such as in Canada, preserving semen could be very helpful to advance the honey production season (Collins 2000b). Since drones take more time to be sexually mature (36 days from egg to sexual maturation) and colonies only start producing them in late spring (May), I.I. can be used with semen collected the prior year to accelerate and elongate the season. Moreover, studies carried out in eastern Canada, where winters are cold and long, demonstrated that the early drones reared in May were less fertile than any other time of the beekeeping season (Rousseau et al. 2015). Therefore, by preserving semen during the winter, semen could be available sooner in spring for I.I. and have better sperm quality from drones reared late the previous summer. Successful sperm preservation is an effective strategy to preserve honey bee genetic diversity, and also to facilitate the selection of lineages tolerant to pests and diseases and prevent further colony loss (Cobey et al. 2013).

1.8.1 Semen Collection

Semen can be collected by one of two methods: (1) directly from the seminal vesicles by dissecting the drone (Mackensen 1955) or (2) by manual eversion of

the endophallus. Collecting semen directly in the seminal vesicles results in more viable sperm, however, it is less practical for I.I., since it takes more time to perform and less semen is collected compared to the manual eversion technique (Collins 2004).

To collect honey bee semen, the endophallus must be manually everted. For the first half of the eversion, the drone is held by the head so that the abdomen is facing upward and a gentle pressure is applied to the thorax with the other hand. Additional light pressures are applied to the base of the abdomen to complete eversion. The creamy coloured semen is expelled out of the endophallus, next to the mucus, and is located at the extremity of the endophallus (Mackensen 1955; Collins 2004). The eversion must be performed in sanitary conditions. It is crucial to avoid contact between semen and the drone's body, using semen that has been mixed with fecal material, and touching the semen (Andere et al. 2011). A sanitary environment reduces semen contamination and ensures that sperm viability is not negatively affected (Andere et al. 2011).

There are many factors that must be considered for sperm preservation such as storage temperature, semen dilution, and the diluent. As early as the 1960s, experiments were conducted with different diluents and temperatures on drone semen (Taber and Blum 1960).

1.8.2 Dilution and Diluent

The diluents used for drone semen preservation are intended to imitate the spermathecal fluid, since sperm stored in the spermatheca maintain high viability for up to several years. Many laboratory diluents can maintain high sperm viability (Moritz 1984; Taylor et al. 2009). Usually, a Tris buffer composed of sugars, amino acids and antibiotics, with a pH of 8.6 is recommended for drone semen preservation (Moritz 1984). Unfortunately, diluting semen can have adverse side

effects on the queen, as several studies have demonstrated that diluted semen can decelerate the beginning of oviposition and fewer sperm are stored in the spermatheca (Kaftanoglu and Peng 1980,1982; Moritz 1984).

1.8.3 Temperature

Drone semen can be stored at room temperature for a few weeks, without losing sperm viability (Cobey 2007; Cobey et al. 2013). The earliest research on drone sperm preservation successfully stored semen at room temperature for four weeks, but bacterial contamination rapidly decreased sperm viability (Taber and Blum 1960). Therefore, by introducing antibiotics to the stored semen, bacterial growth was reduced and semen could be stored at room temperature for 3-4 months (Poole 1969).

Storage below 10˚C and above 32˚C rapidly decreases sperm viability (Taber and Blum 1960; Harbo and Williams 1987), however, spermatozoa can tolerate a brief, one-hour storage at 40˚C (Hopkins and Herr 2010). The optimal temperature for two days storage is 21˚C but semen can be stored between 13 and 25˚C (Harbo and Williams 1987; Locke and Peng 1993). Several studies have reported 70% to 80% sperm viability after storage at 12˚C or 25˚C for 6 weeks (Locke and Peng 1993; Collins 2000b). Semen quality was better preserved at 15˚C than at 24˚C for a storage period of 13 weeks (Poole and Taber 1970). Furthermore, 65% sperm viability was reported after 39 weeks at 12℃, which is acceptable for I.I. (Collins 2000b,a). In 2015, we observed that more sperm preserved at 12-15℃ survived than at 25℃, and our best protocol tested resulted in 47% sperm survival after 17 weeks (Paillard, unpublished data).

Although drone sperm storage is clearly achievable at above-freezing temperatures, it is inadequate for long-term storage since sperm rapidly loses viability after few weeks. Therefore, many researchers have turned to

cryopreservation as a solution for long-term sperm storage.

1.9 Cryopreservation

As for many mammalian species, cryopreservation can be a good method for long-term sperm storage. Cryopreservation is a technique that freezes and conserves live cells and tissues for long periods at very low temperatures, usually in liquid nitrogen (-196°C). Semen cryopreservation has only been reported on few insect species such as the silk moth *Bombyx mori* (Takemura et al. 2000) as well as embryo cryopreservation in *Drosophila melanogaster* (Gardner et al. 1990) and *Spodoptera exigua* (Luo et al. 2006). Frozen semen could be very useful for honey bee I.I. by having access to sperm year round.

Since the late 1970's and early 1980's, multiple honey bee semen cryopreservation techniques have been tested with variable but relatively poor results (Mel'Nichenko and Vavilov Yu 1976; Harbo 1977,1983; Kaftanoglu and Peng 1984). Despite good sperm viability and motility after freezing-thawing, fertility was highly reduced (Harbo 1983; Kaftanoglu and Peng 1984; Cobey et al. 2013) and queens inseminated with frozen-thawed semen did not perform as well as queens inseminated with fresh semen (Peng et al. 1992). Normally, ideal worker production by a queen is approximately 95-99%, but queens inseminated with frozen-thawed semen produced fewer than 50% of workers and the remainders were drones (unfertilized eggs) (Mel'Nichenko and Vavilov Yu 1976; Harbo 1977,1979b,a,1983; Kaftanoglu and Peng 1984; Hopkins et al. 2012).

Many aspects of the cryopreservation protocol can cause sperm damage such as the cryoprotectant used and freezing rate. Rapid freezing and thawing can damage the cell membrane, the acrosome, the nucleus, the flagellum, and split the flagellar mitochondria (Peng et al. 1992). Furthermore, damage to the sperm genome can

occur (Harbo 1981). Eggs fertilized with frozen-thawed sperm had lower viability and emerging drones from the first generation were mosaic, meaning that cells from within one individual had different genetic makeups (Harbo 1979b,1980). The proposed explanation is that the pronucleus of both the spermatozoa and the egg did not fuse properly, hence, both pronuclei developed into independent, haploid tissues (Harbo 1980).

Only recently has drone semen cryopreservation yielded promising results (Taylor et al. 2009; Hopkins and Herr 2010; Hopkins et al. 2012; Wegener and Bienefeld 2012; Wegener et al. 2012; Wegener et al. 2014a). Hopkins (Hopkins et al. 2012) successfully froze drone semen and inseminated queens to obtain a second-generation of queens that were inseminated with the same frozen-thawed semen. Two of the five initial queens inseminated with frozen semen produced a majority of workers. Six of ten first-generation queens inseminated with the same frozen semen laid eggs but only three produce a majority of workers. From one queen, 14 second-generation queens were produced. A third generation was interrupted due to seasonal conditions. These results provide “proof of concept” that honey bee sperm cryopreservation is possible.

Several key factors such as dilution/diluents, cryoprotectants, freezing rates, and thawing are important for successful cryopreservation of honey bee sperm.

1.9.1 Dilution and Diluent

Apart from the cryoprotectant, the choice and the quantity of the diluent added to the drone semen can influence cryopreservation. A higher semen dilution (> 1:3 semen: diluent) helps the spermatozoa to survive during cryopreservation and can be used to inseminate more queens (Taylor et al. 2009). However, diluting sperm increases their motility (Lensky and Schindler 1967), and sperm longevity is often

reduced after motility is activated. Indeed, sperm motility can be higher after cryopreservation than fresh semen, yet result in poor queen fertility after insemination (Kaftanoglu and Peng 1984; Wegener et al. 2014a). The dilution ratio most frequently used is 40% semen and 60% diluent, usually Kiev solution (0.3 g glucose, 0.41 g potassium chloride, 0.21 g sodium bicarbonate, and 2.43 g sodium citrate dihydrate in 100 ml distilled water with 0.05% di-hydrostreptomycin), containing 10% cryoprotectant (Kaftanoglu and Peng 1984).

Hen's egg yolk is often used in diluents for cryopreservation, because it protects cells from cold shock (Amann and Graham 1993). The high concentration of cholesterol in egg yolk helps sperm to survive freezing (Bergeron and Manjunath 2006). However, its composition is not uniform and can vary. Egg yolk also increases the risks of contaminating the semen (Bergeron and Manjunath 2006). Replacing the egg yolk with a synthetic solution (10% w/v cholesterol solution dissolved in 95% ethanol and 20% w/v BSA solution) resulted in low fertility after insemination (Hopkins et al. 2012). Sperm motility after thawing was lower than fresh semen and queens produced mainly drones (unfertilized eggs).

1.9.2 Cryoprotectant

Sperm are stored in liquid nitrogen (-196˚C) thus; sperm need to be mixed with a cryoprotectant to protect from freezing damage. Cryoprotectants prevent ice crystal formation inside the cell by reducing the water freezing temperature (Hopkins and Herr 2010). Many cryoprotectants have been tested on drone semen, including dimethyl sulfoxide (DMSO), glycerol, methyl sulfoxide (MeSO), dimethyl formamide, 1,3-propane diol, 2,3-butane diol, and ethylene glycol (Harbo 1977,1979a; Kaftanoglu and Peng 1984; Hopkins and Herr 2010; Wegener and Bienefeld 2012; Wegener et al. 2014a). Hopkins (2010) compared many and demonstrated that sperm preserved with ethylene glycol for one hour lost motility and had lower viability compared to sperm preserved with DMSO (90% vs. 95%

respectively). He also demonstrated that glycerol killed the most sperm. Ironically, glycerol is commonly used for mammalian semen freezing (Curry 2007). Wagener (2012) found that even if short-term toxicity appeared to be low for most of the cryoprotectants tested (DMSO, 1,3-propane diol, 2,3-butane diol, ethylene glycol, and dimethyl formamide), there was a significant reduction of sperm migration into the spermatheca after I.I..

Hopkins (2010) reported that DMSO was the best cryoprotectant to preserve honey bee semen and was the least toxic compared to the other cryoprotectants. DMSO has been widely used for drone semen cryopreservation since 1976 (Harbo 1977; Wegener and Bienefeld 2012) and maintains good sperm viability (Harbo 1977; Taylor et al. 2009; Hopkins and Herr 2010; Hopkins et al. 2012). Harbo (1977) also demonstrated that 10% DMSO did not affect the quantity of sperm reaching the spermatheca. However, DMSO has been reported to be toxic for both the spermatozoa and the queen after I.I. (Harbo 1986; Wegener and Bienefeld 2012). Hopkins (2010), however, demonstrated that 15% DMSO, highly decreased sperm viability and DMSO can damages the sperm genome through breakage of the chromatids (Kapp and Eventoff 1980). Effectively, 3% of queens' progeny following I.I. with sperm diluted with DMSO, laid eggs that did not hatch, which is a very rare phenomenon occurring in honey bees (Hitchcock 1956; Harbo 1986). Genetic damage occurs in treated spermatozoa (F0), which disrupts the meiotic division in a F1 queen and results in sterile F2 female honey bees (Harbo 1986). However, since it only affected 3% of queens were affected, Wegener & Bienefeld (2012) concluded that the impact of DMSO was less severe than previously thought.

1.9.3 Freezing Rate

It is crucial to freeze semen at an optimal rate; otherwise sperm undergo "cold shock", which means that cells are damaged by the very rapid cooling (Robertson et al. 1990). The ideal sperm-freezing rate differs among species due to sperm

size, morphology, metabolism, and within their membrane phospholipid composition (Barbas and Mascarenhas 2009). Some species require rapid freezing, while others need slow freezing rates (Hopkins and Herr 2010). For honey bee sperm, controlled slow cooling rates yields good sperm viability and motility after thawing (Harbo 1979a; Kaftanoglu and Peng 1984; Hopkins and Herr 2010). By slowly cooling the semen, ice crystals are formed outside the sperm and dehydrates the interior of sperm cells. Thus, ice crystals do not form inside the cells (Mazur 1963).

Early attempts at bee semen cryopreservation using slow cooling of diluted semen (40% semen and 60% Kiev solution, containing 10% DMSO) at 3-4˚C/min to obtain good sperm motility after thawing, however, inseminated queens with the frozen-thawed semen produced fewer than 50% workers (Kaftanoglu and Peng 1984). More recently, a potential freezing rate for drone semen cryopreservation was established (Hopkins and Herr 2010), during which semen is initially cooled to 4˚C over 2 h. The semen is then slowly frozen at a rate of 3˚C/min using a programmable freezer until -40˚C. The semen is then directly plunged into liquid nitrogen. For semen diluted in Harbo's diluent (Harbo 1983), egg yolk and 10% DMSO (ratio of 3:2; semen to diluent), over 90% sperm viability was reported after six days at -196˚C (Hopkins and Herr 2010). However, the fertility of the frozen-thawed semen was not assessed.

1.9.4 Thawing

Thawing protocols are variable in the literature depending on the study. Thawing temperatures vary between 25˚C and 40˚C, and usually requires less than 1 minute, mostly few seconds (Harbo 1979a; Peng et al. 1992; Taylor et al. 2009; Hopkins and Herr 2010; Wegener and Bienefeld 2012). Rapid thawing and temperatures above 40˚C is to be avoided on drone sperm (Peng et al. 1992; Hopkins and Herr 2010). Relatively little research regarding the impact of thawing

rate on bee sperm biology and fertility capacity has been conducted. In some mammals, however, thawing protocol does appear to influence post-thaw parameters (Senger 1980; Nur et al. 2003).

1.10 Centrifugation

Centrifugation of cryopreserved drone semen is useful prior to I.I., to concentrate diluted sperm, homogenize sperm from multiple males and remove cryoprotectants from frozen-thawed sperm (Wegener and Bienefeld 2012; Wegener et al. 2014b). Drone sperm, however, are fragile and centrifugation can damage the cell membrane, causing lethal or sub-lethal injury (Moritz 1984; Harbo 1990; Collins 2004; Cobey 2007; Wegener et al. 2014b). Consequently, only small amounts of centrifuged sperm reach the spermatheca, resulting in poor fertility after I.I. (Kaftanoglu and Peng 1980; Fischer 1987).

More recently, however, worker brood has been successfully obtained after I.I. with centrifuged, unfrozen drone semen (Wegener et al. 2014b). There was no significant difference between the fertility of queens inseminated with centrifuged semen versus fresh semen. This study did not address the use of centrifugation of cryopreserved semen, however, the success of this technique suggests that it could be an effective strategy for post-thaw cryoprotectant removal.

1.11 Problematic

For the last decade, the bee population worldwide has been declining due to many factors such as natural habitat destruction, parasites, pesticide exposure, starvation, transport stress, poor queen health, and winter colony losses (Stokstad 2007; Vanengelsdorp et al. 2009; Moritz and Erler 2016). Colony loss ranges from 35% to 75%, depending on the country (Stokstad 2007). The combination of

multiple factors increases the harm to both honey bees and wild pollinators. The lack of food diversity, due to monoculture agriculture, added to the presence of unintentional pesticide exposure makes the honey bees more vulnerable to various pathogens (Goulson et al. 2015). The most devastating parasites to honey bees in North America include the Varroa mite, and *Nosema ceranea* (fungus) (Bromenshenk et al. 2010; Currie et al. 2010; Neumann and Carreck 2010; Ratnieks and Carreck 2010). Furthermore, many indigenous honey bees have been replaced by commercial stock (domesticated honey bees) which has reduced the genetic diversity of *Apis mellifera* subspecies. Finally, winter colony losses are relatively high. Whereas average winter mortality is approximately 15%, the winter loss average has been 20-25% in Canada and the province of Québec between 2006 and 2015 (Leboeuf et al. 2015).

The global honey bee colony loss has a direct impact on genetic diversity. Successful sperm cryopreservation is an effective strategy to preserve honey bee genetic diversity, and to contribute to the selection of lineages tolerant to pests and diseases and prevent further colony loss (Cobey et al. 2013).

1.12 Project Objectives

The main goal of this project is to evaluate different techniques of drone semen preservation using sperm viability and fertility after instrumental insemination as end points. Very few studies have been conducted on comparing the efficiency of long-term drone semen preservation at above freezing temperatures versus frozen storage. Moreover, although cryopreservation of semen is a well-established strategy in many species, the cryoprotectants used are toxic to the queen after instrumental insemination. We therefore to evaluated the efficacy of semen centrifugation after preservation to eliminate the cryoprotectant prior to I.I.

Specific objectives:

- Determine which semen preservation temperature (16˚C vs. -196˚C) is better for 1 year storage.
- Determine if semen centrifugation to eliminate the cryopretectant after freezing-thawing will improve fertility after instrumental insemination.

1.13 Hypotheses:

1. Cryopreservation is more effective than fresh preservation at 16˚C for storage of honey bee sperm for periods up to one year.
2. Post-thaw centrifugation to remove cryoprotectants from the sperm will improve fertility after I.I.

Chapter II

Preservation of Honey Bee (*Apis mellifera* L.) Semen

This manuscript has been submitted to periodical ***The Canadian Entomologist*** for publication. The first author is Marilène Paillard. Co-authors are Andrée Rousseau M.Sc. (Centre de recherche en sciences animales de Deschambault), Janice L. Bailey Ph.D. (Centre de recherche en reproduction, développement et santé intergénérationnelle, Université Laval) and Pierre Giovenazzo (Département de biologie, Université Laval).

2.1 Résumé

La conservation du sperme d'abeille domestique (*Apis mellifera* Linnaeus) est un outil important pour sauvegarder leur diversité génétique. Notre objectif général est de développer une méthode de conservation de la semence d'abeille. Deux protocoles de recherche ont été réalisés. L'hypothèse no.1 était que la cryoconservation (-196°C) de la semence est plus efficace à long terme que la température 16°C. Suite à une conservation d'un an, nos résultats montrent que les spermatozoïdes congelés étaient plus viables que ceux conservée à 16°C. Le cryoprotectant utilisé pour congeler la semence (diméthylsulfoxyde, DMSO) pourrait nuire à la fertilité de la reine suite à l'insémination instrumentale. L'hypothèse no.2 était que la centrifugation de la semence éliminerait le DMSO et améliorerait la fertilité de la reine après l'insémination. Nos résultats démontrent que la centrifugation n'affecte pas la viabilité des spermatozoïdes. Nous n'avons pu conclure, dû à la variabilité des résultats, si la centrifugation du sperme améliorait la fertilité des reines suite à l'insémination.

2.2 Abstract

Preservation of honey bee (*Apis mellifera* Linnaeus) sperm, coupled with instrumental insemination, is an effective strategy to protect the species and their genetic diversity. Our overall objective is to develop a method of drone semen preservation; therefore, two experiments were conducted. Hypothesis #1 was that cryopreservation (-196˚C) of drone semen is more effective for long-term storage than at 16˚C. Our results show that after 1 year of storage, frozen sperm viability was higher than at 16˚C, showing that cryopreservation is necessary to conserve semen. However, the cryoprotectant used for drone sperm freezing, dimethyl sulfoxide, can harm the queen and reduce fertility after instrumental insemination. Hypothesis #2 was that centrifugation of cryopreserved semen to remove DMSO prior to insemination improves fertility. Our results indicate that centrifuging semen does not affect sperm viability. We cannot yet confirm that centrifugation improves queen health and fertility after insemination due to variable results after instrumental insemination.

2.3 Introduction

Honey bees (*Apis mellifera* Linnaeus; Hymenoptera: Apidae) are critical players in the agricultural industry for food production as they account for the vast majority of managed insect pollination services. In the last decade, however, there have been dramatic worldwide losses of honey bee colonies caused by numerous factors such as natural habitat destruction, parasites, pesticide exposure, poor food supply, transport stress associated with colonies import/export, poor queen health (Pettis et al. 2016), and winter colony losses (Stokstad 2007; Vanengelsdorp et al. 2009). These losses are a major threat to honey bee diversity and preservation of honey bee sperm is an effective strategy to protect their genetic diversity and also contribute to selective genetic breeding (Collins 2000b).

As early as the 1960s, the influence of different diluents and temperatures on drone semen has been investigated (Taber and Blum 1960). Fresh drone semen can be stored at above freezing temperatures for a few weeks without losing sperm viability (Cobey 2007; Cobey et al. 2013). Drone semen can be stored between 13˚C and 25˚C (Harbo and Williams 1987), however, sperm viability seems to be higher when stored between 12˚C and 16˚C. Sperm viability of 70% - 80% after storage at 12˚C for 6 weeks has been reported (Locke and Peng 1993; Collins 2000b). Furthermore, studies demonstrated higher sperm viability after 13 weeks storage at 15˚C than at 24˚C (Poole and Taber 1970). Good sperm viability (65%) was obtained after 39 weeks preserved at 12℃ (Collins 2000b), which is acceptable for instrumental insemination (I.I.) and it is, probably, to our knowledge, the maximum period of storage for adequate fertility (Collins 2000b,a).

As for many mammalian species, cryopreservation can be used for the long-term sperm storage (Bailey et al. 2003; Curry 2007; Blackburn et al. 2009). Having access to cryopreserved sperm permits insemination at any time of the year. Furthermore, for bee production, it is easier to transport cryopreserved honey bee

semen instead of live drones for I.I. and also reduces the risk of spreading bee pathogens (Cobey 2007). Cryopreservation of honey bee drone semen, however, is not optimized for commercial applications. Research in the 1970s-1980s, tested multiple techniques without marked success, resulting in highly reduced fertility, even though post-thaw sperm motility and viability was good (Mel'Nichenko and Vavilov Yu 1976; Harbo 1977,1979b,a,1983; Kaftanoglu and Peng 1984; Peng et al. 1992). A recent report describes successful drone semen cryopreservation resulting in a second-generation of queens using I.I. with the same frozen-thawed semen (Hopkins et al. 2012). This report provided an important "proof of concept" that honey bee sperm cryopreservation is possible. Alternatively, the cryoprotectants used in this approach, including dimethyl sulfoxide (DMSO) and hen's egg yolk, which are commonly used to freeze mammalian cells, are thought to be toxic to sperm and/or the queen reproductive tract.

Centrifugation of cryopreserved drone semen is useful prior to I.I., to concentrate diluted sperm, homogenize sperm of multiple males and remove cryoprotectants from frozen-thawed sperm (Wegener and Bienefeld 2012; Wegener et al. 2014b). Drone sperm, however, are fragile and centrifugation can damage the cell membrane, causing lethal or sub-lethal injury (Moritz 1984; Harbo 1990; Collins 2004; Cobey 2007; Wegener et al. 2014b). Consequently, only small amounts of centrifuged sperm reach the spermatheca, resulting in poor fertility after I.I. (Kaftanoglu and Peng 1980; Fischer 1987). More recently, however, worker broods has been successfully obtained after I.I. with centrifuged, unfrozen drone semen (Wegener et al. 2014b). There was no difference between the fertility of queens inseminated with centrifuged semen versus fresh semen. This study did not address the use of centrifugation of cryopreserved semen, however, the success of the technique suggests that it could be an effective strategy for post-thaw cryoprotectant removal.

The main goal of this project is to evaluate different techniques of drone semen preservation using sperm viability and fertility after instrumental insemination as

end points. In this research, we determine what best semen preservation temperatures (16˚C and -196˚C) is better for 1 year storage, and if semen centrifugation to eliminate the cryoprotectant after freezing-thawing will improve fertility after instrumental insemination.

2.4 Materials and Methods

2.4.1 Drone Management

Honey bee colonies were housed at the Centre de Recherche en Sciences Animales de Deschambault (CRSAD; 46˚ 40' 26.85''N, 71˚ 54' 54.39'' W), Deschambault, Canada, and the laboratory work was conducted at Université Laval, Québec, Canada. Mature drones were obtained from honey bee colonies with open-mated queens of the same genetic lineage of Italian stock and Buckfast stock from the CRSAD breeding program. During August 2014, over 5 000 drones of approximately 20 days of age were randomly collected from hives. Drones were captured from the side frames of the brood chamber or at the entry of the hive when they arrive from an orientation flight (Cobey et al. 2013; Rousseau et al. 2015). Immediately before collecting semen, drones were placed in a handmade illuminated lab cage (30- by 30- by 30- cm wooden cage with 1/8-inch mesh screen) where they were able to fly and defecate, which helps initiate semen ejaculation (Rousseau et al. 2015, Collins 2004).

2.4.2 Semen Collection

Semen was collected using a Harbo Large Capacity Syringe (Model GS 1100, Fisher Scientific Ltd., Ottawa, Canada) (Harbo 1985) after performing the manual eversion technique (Cobey et al. 2013) All syringe parts were sterilized with 70% alcohol and rinsed with distilled water. The syringe was attached to a 50 µl capillary with a thin, handmade glass tip to collect semen. The syringe was filled with sterile antibiotic saline (10 g sodium chloride in 1000 ml distilled water with 0.25% (v/v) dihydrostreptomycin). All chemicals were purchased from Sigma Chemical Co. (St. Louis, MO). An air space between the saline solution and the semen was added in the capillary to avoid mixing the two fluids. In total, 2000 µl semen were collected over three days and held at room temperature before experimentation. The 2000 µl

semen were collected into 40 capillaries by two different persons. The 40 semen filled capillaries were randomly expelled into 1.5 ml sterile Eppendorf tubes to produce 6 different pools (n=6).

2.4.3 Semen Dilution

Each semen pool was divided into four different treatments: 1) fresh semen preserved at 16˚C without centrifugation, 2) fresh semen preserved at 16˚C with centrifugation, 3) semen cryopreserved at -196˚C without centrifugation, and 4) semen cryopreserved at -196˚C with centrifugation.

For fresh semen preserved at 16˚C, semen was diluted 1:1 (vol/vol) with the Hopkins' modified extender solution (Table 2.1) (Hopkins et al. 2012). For cryopreservation, semen was first diluted with the Hopkins modified extender (5:1 semen: diluent; vol/vol). The diluted semen was further extended with a cryoprotecting solution, Harbo's diluent (Table 2.1; 3:2 diluted fresh semen: Harbo's diluent; vol/vol). The fully diluted semen of each treatment was gently mixed with the tip of a micropipette and then recollected into sterile microdispenser capillaries (one capillary/treatment/pool). Each semen pool (n = 6) was divided into four treatments of approximately 80 µl of diluted semen in a single capillary. In total, 24 capillaries of diluted semen were prepared (4 treatments X 6 pools). Furthermore, one extra capillary of approximately 20 µl of diluted semen was prepared to evaluate sperm viability at Days 90 and 180 of storage for each temperature (2 capillaries/temperature/pool).

2.4.4 Fresh Semen at 16˚C

For preservation at 16˚C, glass capillaries were used to store the semen. The capillaries contained the diluted semen in the middle, a small amount of extender at each extremity end (to prevent semen desiccation), and were sealed with Critoseal® at each end (Burley et al. 2008). An air bubble separated the liquids between. The capillaries were stored shielded from light in a Thermofix incubator at 16˚C ± 1˚C.

2.4.5 Cryopreservation at -196˚C

The semen that was fully diluted in Hopkin's modified extender and Harbo's diluent was stored in 0.25-ml plastic freezing straws (IMV Technologies). A small quantity of Hopkins's modified extender was added at the extremities of the straws, separated from the diluted semen by an air space. Straws were sealed with an Ultraseal 21 heat sealer (Minitube, Germany). The diluted semen was submerged vertically in a room temperature water bath (500 ml) and transferred into a walk-in refrigerator at 4˚C for two hours to avoid thermal shock to the sperm. The diluted semen was then transferred into straws and they were deposited into a programming freezer (Cell Freezer R204; Diamond Lab Supplies Inc., Mississauga, Ontario, Canada), which cooled from 4˚C to -40˚C (-3˚C/min). Finally, samples were rapidly transferred into liquid nitrogen. When required, the frozen semen was thawed by plunging straws in water at 37˚C for 30 seconds.

2.4.6 Centrifugation

After storage, half of the semen samples stored at 16˚C and -196˚C were transferred in 1.5 ml sterile Eppendorf tubes and centrifuged for 10 minutes at 1000 *g* (Wegener et al. 2014b). The supernatant was removed and the

concentrated sperm pellet was re-diluted 1:1 with Hopkins' modified extender. Semen samples were recollected into new glass capillaries, in preparation for I.I.

2.4.7 Instrumental Insemination

Queens, reared in queenless colonies (Buchler et al. 2013) were from the same genetic lineage, which was different from that of the drones. Three queens were inseminated at 6-8 days of age with diluted semen of each of the four treatments (preserved at 16˚C and cryopreserved with or without centrifugation) from each pool (n = 6; total of 72 queens; 3 queens x 4 treatments x 6 pools). One treatment of 1 to 4 minutes exposed to CO_2 was given to the queen the day before insemination (Cobey et al. 2013). Each queen was inseminated with 8-10 µl of stored sperm, transferred individually into small cages and stored in queenless colonies. The queens were euthanized by placing them in the freezer (-20˚C) for 15 minutes. Sperm migration is complete approximately 40 h after mating (Woyke 1983). Therefore, queens were dissected 48h post insemination to collect their spermathecae for sperm count and viability. The spermatheca was removed from the queen's abdomen, placed in 0.5 ml Hopkins' modified extender solution, and opened with forceps to release the sperm. Sperm count and viability were assessed using methods described below.

2.4.8 Sperm Viability

Sperm viability was assessed for each pool before storage and for each treatment at 90, 180, and 330 days after storage at 16˚C and cryopreservation. Sperm viability was evaluated using the Live/Dead Sperm Viability Kit (L-7011; Life Technology Inc., Burlington, Ontario, Canada), which is composed of SYBR-14 and propidium iodide (PI) (Collins and Donoghue 1999). A volume of 1 µl of semen of each group was suspended in 500 µl modified Kiev solution (MKS) (0.3 g glucose, 0.41 g potassium chloride, 0.21 g sodium bicarbonate, and 2.43 g sodium

citrate dihydrate in 100 ml distilled water with 0.05% dihydrostreptomycin) (Kaftanoglu and Peng 1984; Locke et al. 1990) to reduce sperm concentration. Ten µl diluted SYBR-14 (1:50 with MKS) and 5 µl PI were mixed with semen on a slide and a coverslip was applied. Sperm were then evaluated using a Motic™ microscope equipped with a red/green fluorescent filter (excitation at 485-517 nm and emission at 536-617 nm) at 400X. Each spermatozoon was scored as either alive (green fluorescence), or, if sperm had lost membrane integrity, dead (red fluorescence). For each slide, 200 sperm were counted per slide as either dead or alive; each semen sample was assessed, in this manner, in quadruplets and reported as the percentage of viable sperm (Burley et al. 2008).

2.4.9 Sperm Count Assessment

Sperm count was evaluated for each pool before treatment and in the queen's spermatheca after I.I. with a Bright-Line haemocytometer (Hausser Scientific, Pennsylavania, USA) (Cobey et al. 2013). A semen volume of 1 µl was diluted in 1 ml MKS (Dilution factor = 1000). Sperm were counted in four center squares (0.1 mm^3 = 0.1 µl) under a light microscope at 400X magnification. To obtain sperm numbers per drone, the following formula was applied:

$$\text{Cells/µl} = \text{average cell count per square} \times \text{dilution factor} \times 10$$

2.4.10 Statistical Analysis

Experiments were set up as a complete randomized design. The six pools per storage temperature (16˚C vs. cryopreservation) were analyzed as unequally spaced repeated measures analysis of variance (ANOVA), using PROC Mixed (SAS Institute 2012-2015; University Edition). Also, orthogonal contrast comparisons were conducted between and within treatments for 0, 90, 180, and

330 days. Multiple comparisons of least squares means were made using a Tukey-Kramer test to compare temperatures and storage days. An arcsine transformation was used on all sperm viability data to meet the homogeneity of variance and the normal distribution.

2.5 Results

2.5.1 Preservation Temperatures

Sperm viability was assessed at various times over 330 days during storage of fresh semen at 16˚C or cryopreservation at -196˚C (Figure 2.1). The mean sperm viability of the 6 pools for the initial day (Day 0 of storage) was 89% ± 2% (mean ± standard error). After 90 days, there was a significant difference between the two preservation temperatures. Cryopreserved semen had less viable sperm than semen stored at 16˚C (77% ± 5% vs. 61% ± 5%; p = 0.0006). No significant difference between the two temperatures was observed after 180 days storage (p = 0.950). After 330 days of storage, all sperm stored at 16˚C were dead while the frozen-thawed semen had a mean sperm viability of 76% ± 5% (p < 0.0001). For both temperatures, sperm viability declined in time ($F_{(3,28)}$ = 838.99; p < 0.0001).

2.5.2 Sperm Centrifugation

Since all stored sperm at 16˚C were dead after 330 days, centrifugation was only evaluated on frozen-thawed semen. There was a tendency for decreased sperm viability due to centrifugation (Figure 2.2; $F_{(1,11)}$ = 4.49; p = 0.0652). Mean sperm viability before centrifugation for the 6 pools was at 78% ± 3%, while the mean sperm viability after centrifugation was 75% ± 3%.

2.5.3 Queen's Spermatheca Assessment

No queens were inseminated with semen stored at 16˚C, since all sperm were dead at the end of storage (330 days). Therefore, to test the hypothesis that centrifugation of semen after storage to remove excess diluent and cryoprotectants would improve fertility outcome, queens were inseminated with frozen-thawed

semen that was either centrifuged or not centrifuged, such that 36 queens were used for I.I. instead of 72 queens. The results varied greatly. For both treatments combined (centrifuged and not centrifuged), 82% of queens were alive 48 h after I.I., however, 13% of these queens had an empty spermatheca. The amount of sperm in the spermatheca and sperm viability varied greatly between queens (Table 3). Sperm count varied between 5,000 and 150,000 and sperm viability varied between 10% and 71%. There was no difference due to centrifuging cryopreserved sperm on queen survival ($F=6.51$; $p=0.1360$), sperm count (Table 3; $F = 1.07$; $p = 0.3230$) or sperm viability ($F = 0.05$; $p = 0.8195$).

2.6 Discussion

The purpose of this study was to (1) determine the best semen preservation temperatures (16˚C and -196˚C) for one-year storage of honey bee sperm and (2) determine if semen centrifugation to eliminate the cryoprotectant after freezing-thawing would improve fertility after instrumental insemination. Our data showed that sperm preserved at both temperatures is viable up to 180 days, but only cryopreserved sperm is viable after 330 days. Cryopreservation is necessary for long-term sperm storage. However, centrifugation of the frozen-thawed semen did not improve fertility after insemination, even though centrifugation did not affect sperm viability.

2.6.1 Cryopreservation for Long-Term Storage

Our hypothesis for Objective 1 was that cryopreservation is more effective than fresh preservation at 16˚C for storage up to one year. Good sperm viability was observed for both treatments (fresh at 16˚C and cryopreserved at -196˚C) after storage for 180 days, 25 weeks (70% ± 8% and 64% ± 8%, respectively). In contrast, after 330 days (47 weeks), semen stored at 16˚C had 100% sperm mortality whereas frozen-thawed semen still had good sperm viability (76% ± 5%). Apart from initial freezing damage to the sperm, cryopreservation maintains sperm parameters almost indefinitely in proper storage conditions (Bailey et al. 2003). Taken together, it appears that fresh honey bee sperm can be effectively stored for 35-39 weeks (Collins 2000b), but viability diminishes to unacceptable levels after this duration. Poole and Taber (1970) reported successful fertilization following I.I. with semen stored at 13-15˚C for 35 weeks. Collins (2000a) also successfully stored semen at 12˚C and 25˚C for 39 weeks (273 days) with a sperm viability of 64% and 45%, respectively. Locke and Peng (1993) also obtained good sperm viability (77.6%) after 6 weeks of fresh preservation at 21-24˚C.

2.6.2 Sperm Centrifugation to Improve Fertility

We hypothesized that eliminating the cryoprotectanst (DMSO and egg yolk) from the stored semen would improve fertility after I.I.; therefore, the thawed semen was centrifuged to remove the freezing extender. Centrifugation did not alter sperm viability, although there was tendency towards lower viability (p=0.065). Our hypothesis, therefore, that centrifugation to remove DMSO would improve sperm quality, was incorrect. Many molecules such as proteins, amino acids and sugars present in the drone's seminal fluid are known to help sperm viability after storage (Boomsma et al. 2009). Proteins in the seminal fluid could help sperm to survive in the queen's oviducts within the 40 h after mating, before sperm are transferred to the spermatheca (Boomsma et al. 2009). Thus, centrifuging the semen may have eliminated essential fluids for sperm viability.

2.6.3 Fertility Parameters after Instrumental Insemination

Our hypothesis was that post-thaw centrifugation to remove cryoprotectants from the sperm could improve fertility after instrumental insemination. Following I.I., 18% queen mortality was observed and limited sperm migration to the spermatheca occurred in queens that survived (13% of those queens), irrespective of centrifugation or not. The surviving queens containing sperm in their spermathecae did not contain enough sperm to be considered fertile. To have productive colonies, queens must have sufficient sperm in their spermatheca to produce sufficient workers for many years. Inseminated queens store less sperm than naturally mated queens (2.1 to 4.8 million vs. 4.1 to 5.9 million) (Wilde 1994). A queen receiving less than 500,000 sperm rapidly becomes a drone layer, which means she only lay unfertilized eggs (Koeniger et al. 2014). However, in our study, much lower sperm numbers were found in the spermatheca after I.I. The highest amount of sperm stored in a spermatheca, in the present study, was approximately 150 000, which is much less than the minimum amount required to consider a

queen fertile. Furthermore, sperm viability in the spermatheca varied greatly and thirteen inseminated queens did not have the minimum sperm viability required (45%) without affecting the worker brood production (Collins 2000a). Therefore, these queens would not have been able to generate a strong commercial colony. On the other hand, inseminated queens could be used to lay a minimum of female eggs that would develop into future queens (F1). Therefore, F1 queens would have the same selected genetics of the inseminated queen and it would accelerate breeding and genetic selection.

Both queen mortality and poor sperm migration are possibly due to the presence of mucus in the semen samples. A previous report tested a washing technique by centrifugation to remove undesirable mucus collected with the semen (Kaftanoglu and Peng 1980; Collins 2003). Un-centrifuged frozen-thawed semen seemed to contain more mucus than semen that was centrifuged after thawing. Therefore, one could speculate that centrifugation of semen would help remove excess mucus that was inadvertently collected with the sperm, hence favouring the outcome of I.I. However, we did not detect any differences in reproductive parameters after I.I. between centrifuged and non-centrifuged semen including queen viability, sperm viability and sperm count in the spermatheca. Much care must be taken to avoid collecting mucus with the semen, because mucus does not normally penetrate the vagina during copulation. Mucus in the queen's reproductive tract appears to clog the oviduct, killing the queen (Cobey et al. 2013). Considerable technical expertise is required for proper semen collection. Furthermore, to our knowledge, the impact of hen's egg yolk, which is in the cryodiluant, on the queen's reproductive tract has not been studied. It is unknown if egg yolk components used in our experiment can clog the sperm duct, promote infection or have an otherwise undesirable impact.

2.6.4 Conclusion

Our results confirm that drone sperm storage is achievable at above-freezing temperatures for up to 180 days; however, it is not a technique adequate for long-term storage since sperm rapidly lose viability after several months of storage. Therefore, cryopreservation is a theoretical solution for long-term sperm storage. Our results also confirm that Hopkins' (2010, 2012) method of cryopreservation is achievable and it is better for long-term sperm storage than above-freezing temperatures for duration of close to a year. Even though fresh sperm stored at 16˚C did not survive after 9 months, more research could lead to improved storage method at above-freezing temperatures. It is an easy, low cost and practical method for overwintering semen that could be used to easily inseminate queens early in spring the next year. We were also able to demonstrate that centrifugation does not markedly affect sperm viability. We were unable to determine, however, if semen centrifugation effectively removes sperm cryoprotectants (DMSO and egg yolk) to improve fertility, since there was too much variability in our results, presumably caused by technical problems in semen collection and excess mucus.

2.7 Acknowledgments

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Table 2.1: Hopkin's modified extender used to dilute semen stored at 16˚C and -196˚C (Hopkins et al. 2012). Products were dissolved in a final volume of 100 ml distilled water.

Penicillin	0.0125 g
Streptomycin	0.011 g
N-[tris(hydroxymethyl)methyl]-2-aminoethanesulfonic acid (TES) buffer	30 mM
Tris base	30 mM
EDTA	0.01 mM
Sodium phosphate dibasic	1 mM
Sodium citrate	1 mM
Glucose	2.7 mM
Arginine	0.57 mM
Glycine	0.1 mM
Proline	4.3 mM
Catalase	0.5 mg
Bovine serum albumin	2 mg
KCl	82 mM
NaCl	83 mM
$NaHCO_3$	5 mM

Table 2.2: Harbo's diluent (DMSO 25% (v/v), egg yolk 25% (v/v), and Buffer 50% (v/v) used for semen cryopreservation (Hopkins et al. 2012).

Dimethyl sulfoxide	250 µl
Egg yolk	250 µl
Buffer *	500 µl

* Buffer: 0.956 g NaH_2PO_4 and 0.449 g Na_2HPO_4 diluted in 25 ml distilled water

Table 2.3: Table representing results of queen I.I. with frozen-thawed semen not centrifuged and centrifuged. Percentage of queen mortality, or sperm found in the spermatheca after I.I. with both treatments ($F_{(3,2)} = 6.51$; $p = 0.14$). Comparison of sperm count ($F_{(1,12)} = 1.07$; $p = 0.323$) and sperm viability ($F_{(1,12)} = 0.05$; $p = 0.82$) in the queen's spermatheca after I.I. for both treatments.

		Treatment	
		Frozen-thawed, not centrifuged **(n = 18 queens)**	Frozen-thawed, centrifuged **(n = 18 queens)**
Inseminated queens	**Queen mortality**	5/18 (28%)	1/18 (5%)
	Spermatheca empty	1/18 (5%)	4/18 (23%)
	Sperm in spermatheca	12/18 (67%)	13/18 (72%)
Spermatozoa found in queens' spermatheca	**Min. sperm count**	5,000	4,750
	Max. sperm count	142,125	148,125
	Min. sperm viability	10%	26%
	Max. sperm viability	68%	71%

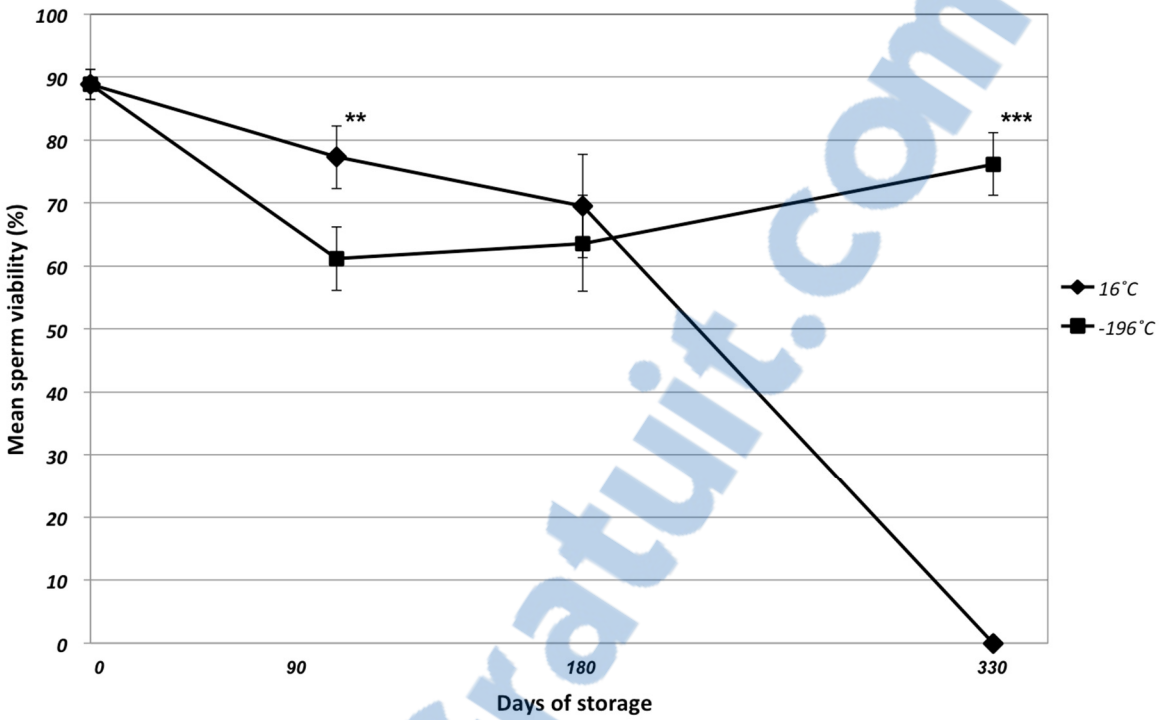

Figure 2.1: Progression of mean sperm viability (± SEM) during 11 months, for two temperatures (16˚C vs. -196˚C) at different evaluation days ($F_{(3,28)}$= 484.27; p < 0.0001). There was a significant difference between the two temperatures at 90 days (** p < 0.001) and 330 days (*** p < 0.0001) but not at 180 days (p > 0.05).

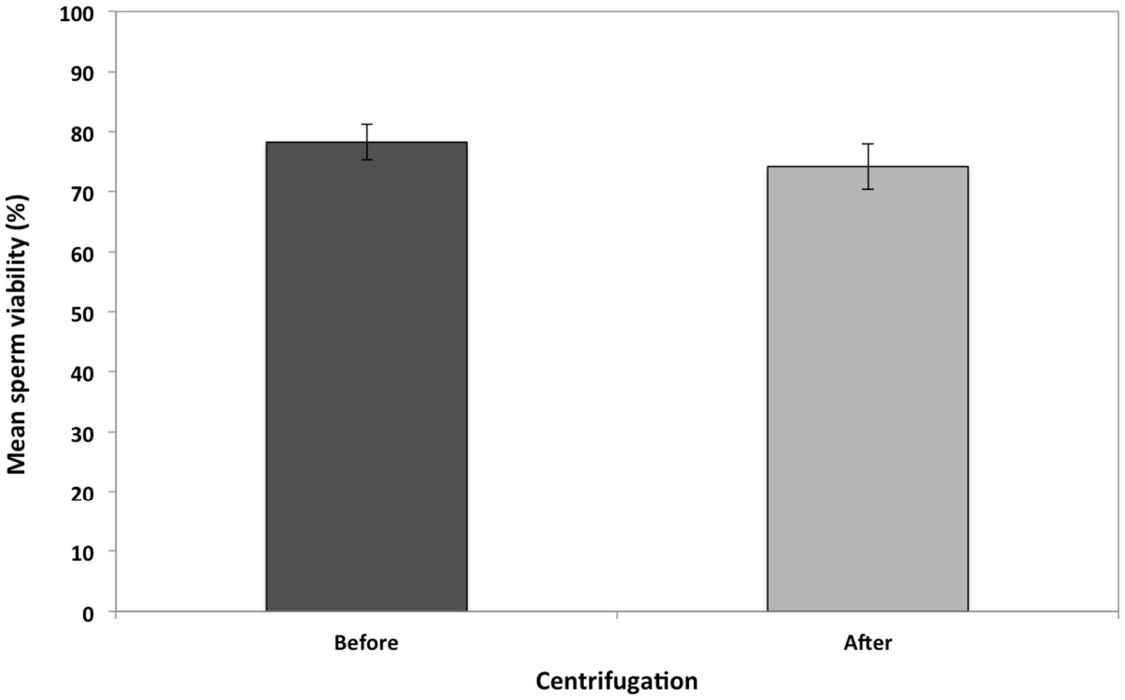

Figure 2.2: Mean sperm viability (± SEM) before and after centrifugation of frozen-thawed semen preserved during 330 days ($F_{(1,11)} = 4.49$; $p = 0.0652$).

Chapter III: General Conclusion

The main goal of this master's research was to evaluate different techniques of drone semen preservation using sperm viability and fertility after instrumental insemination as end points. This experiment helped us to better understand the value of cryopreservation to preserve honey bee semen, but also that there are still many challenges to overcome before achieving successful instrumental insemination with cryopreserved semen.

The first stated hypothesis of this master's thesis is that cryopreservation will be more efficient for semen preservation for one year compared to fresh semen stored at 16˚C. Effectively, this research demonstrated that frozen semen can be preserved much more effectively than at above freezing temperatures (16˚C). The sperm viability of freezing semen after one year was very good (76% ± 5%). Hopkins' method of cryopreservation is achievable, and it is better for long-term storage than above-freezing temperatures. However, even though sperm stored at 16˚C were dead after one year, good sperm viability was observed after 180 days at levels higher than accepted sperm viability threshold necessary for fertility (45%) (Collins 2000a). Storing semen at above-freezing temperatures could be a low cost, practical method to over winter and then be able to inseminate queens early in spring the next year.

The second hypothesis stated that centrifugation of semen will prevent the cryoprotectant's toxicity from affecting the queen's health and fertility after instrumental insemination. We could not prove if this hypothesis was true or not. We can stipulate that centrifugation does not affect sperm viability, but we were not able to determine if centrifugation really helped the cryopreservation technique by removing the cryoprotectant and, thus, improving the queen's health and fertility after insemination. Queen mortality after I.I. was high, irrespective of treatment, and sperm migration to the spermatheca was not observed in many surviving queens. Small amounts of mucus were present in the semen samples, which many have contributed because to the poor results of the I.I. Therefore, we could not conclude if there was any treatment effect on reproductive outcome.

This project was carried out based on the lack of scientific research on comparative drone semen preservation temperatures: freezing and above freezing. Furthermore, little research has been done on centrifugation, and we wanted to understand more the impact of this technique on drone sperm. This project brought us more questions about semen preservation and queen insemination then answers to our questioning. DMSO is still a problem for both sperm and queens. Wagener and Bienefeld (2012) proposed a mixture of two cryoprotectants to reduce toxicity. Mixing trehalose with DMSO could be a good solution, since trehalose is often found in insects as a natural cryoprotectant (Lee 1991) and it is also present in the honey bee seminal plasma (Blum et al. 1962). Furthermore, to our knowledge, the impact of the egg yolk, found in the cryodiluant, into the queen's reproductive has not yet been studied. We do not know if this substance can clog in the sperm duct or if it can promote infection. This could be an essential aspect to successful queen insemination. We need to emphasise on developing a synthetic solution that could replace egg yolk and avoid the variability composition of egg yolk. Finally, many washing techniques, for example migration-based techniques or glass wool filtration, are well established for mammal semen that could be tested on drone semen to eliminate the cryoprotectant, but to also eliminate possible mucus in the semen.

Semen collection and instrumental insemination are both techniques that need a lot of practice to be perfected. These techniques are well established, for decades now, all around the world, but results can reflect the expertise, meaning that best results will come with practice.

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