Impact of the Age on Early Embryonic Mortality (EEM) and Embryo Quality in the Honey Bee (*Apis mellifera* L.)

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Abstract

This study on the honey bee, *Apis mellifera*, consists of three major parts. The first involves the characteristics of the spermathecal content of old and young honey bee queen. The second examines maternal age effects on embryonic mortality and juvenile development of offspring in the honey bee. The third investigates on the impact of semen age on early embryonic mortality, embryo quality and larvae development in the honey bee.

Semen collected from the spermatheca of old queen bees show different sperm movement patterns and slower speed than sperm from the spermathecae of young queens. This ability is possibly related to different enzyme activities and metabolisms found in the spermathecal contents of differently aged queens.

The embryonic development and larval growth rate have been examined with regard to queen honey bees of different ages (2-year-old to freshly mated queens) during two years (2005 and 2006). Early embryonic mortality “EEM” has been found to be higher within the eggs from old queens than in those from younger queens. Egg volume, consequently embryo size, reduces as queen’s age.

A further investigates embryonic mortality in offspring originating from older semen. This has been carried out by extracting the semen from the spermatheca of an old or/and young mated queen and re-inseminating it into a virgin queen, in order to adjust for queen age. The investigation show higher embryonic mortality in the offspring from virgin queens inseminated with semen extracted from older queens than with semen from younger queens. The relative percentage of early and late embryonic mortality within the groups was different between queens re-inseminated with aged semen. High embryonic mortality in the control semen ages may be affected by the method of extracting from semen out of the spermatheca and re-inseminating it into a virgin queen. Empty egg phenomenon, which has been found in both groups, may be related to this technique.

Keywords:

Enzyme activity, Early embryonic mortality, Embryogenesis, Maternal Effect

Zusammenfassung

Die vorliegende Studie über den Alterseinfluß bei der Honigbiene (*Apis mellifera)* besteht aus drei Hauptteilen. Der erste Teil befasst sich mit den Charakteristiken des Spermathekeninhalts alter und junger Bienenköniginnen. Im zweiten Teil geht es um die Auswirkungen des maternalen Alters auf die embryonale Mortalität und die juvenile Entwicklung der Brut. Im dritten Studienteil werden die Auswirkungen der Verweildauer in der Spermatheca auf die embryonale Mortalität, Embryonenqualität und Larvenentwicklung der Nachkommen untersucht.

Der Samen aus den Spermatheken älterer Bienen weist andere Bewegungsmuster, eine geringere Geschwindigkeit und eine anderen Enzymaktivität auf als der Samen, der aus den Spermatheken junger Königinnen gewonnen wurde. Ein altersbedingtes Nachlassen der Fertlität ist daraus zu erklären.

Im Laufe von zwei Jahren wurde die embryonale Entwicklung und das Larvenwachstum von Nachkommen unterschiedlich alter Königinnen untersucht (2-jährige bis frisch begattete Königinnen). Ältere Königinnen legten kleinere Eier und deren Nachkommen zeigten eine signifikant höhere Mortalitätsrate und kleinere Entwicklungsstadien als die Nachkommen jüngerer Königinnen.

In einer weiteren Studie wurde die embryonale Mortalität und die Embryonalentwicklung von Nachkommen, die aus älteren Samen entstanden, untersucht. Dabei wurde der Samen aus den Spermatheken von alten und jungen begatteten Königin entnommen und jungfräuliche Königinnen übertragen. um das Alter der Königin auszugleichen. Bei der Untersuchung wurde eine höhere embryonale Mortalität und generell, zu gewissen Entwicklungszeiten signifikant, kleine Entwicklungsstadien bei den Königinnen festgestellt, die mit dem älteren Samen befruchtet wurden. Der relative Anteil an früher und später embryonaler Mortalität war auch zwischen den beiden Spermien-Alterlassen signifikant unterschiedlich. Die insgesamt hohe embryonale Mortalität auch in der Kontrollgruppe (Königinnen besamt mit Sperma aus den Spermathecen junger Königinnen) belegt, dass die Methode der Samenextraktion und Reinsemination einen großen Einfluß auf die Embryonalentwicklung hatte. Auch das Phänomen

„leerer Eier“, welches in beiden Gruppen in gleicher Frequenz vorgefunden wurde, ist möglicherweise durch diese Methode bedingt.

Schlagwörter:

Enzymaktivität, embryonale Mortalität, Embryonalentwicklung, Maternale Effekte

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1 General Introduction

1.1 General Introduction

Hymenopteran societies are characterised by the reproductive division of labour among females (queens and workers). The first caste consists of the queen whose initial activity is the mating ritual during which sperm are deposited in her spermatheca, a storage receptacle. Reproduction in these species involves the long-term storage of sperm in spermathecae, the spermatozoa being released progressively from the spermatheca to fertilise the eggs. Inside the spermatheca, sperm may live for weeks, months, or even years in long-lived species (Taber and Blum, 1960; Neubaum and Wolfner 1999). In the honeybee (*Apis mellifera*), queens can survive for up to five years, storing sperm in the spermatheca during this period and laying up to 2000 eggs per day. When production levels naturally drop, the old queen either leaves the nest to start a new colony (swarming) or she is killed by the workers. The second caste in eusocial insects is the worker. Each colony has dozens to millions of workers, which have a low or even zero reproductive potential and perform other tasks necessary for their colony to survive (Keller 1993). Male honeybees usually do not take part in colony life, their main task being to mate with the queen. Mating normally occurs outside the nest (drone congregation area) with unrelated queens. Drones mate during a limited time window of 3 - 6 weeks.

Social insects generally produce a large number of offspring and the efficiency of a colony of these insects strongly depends on the fertility of the mother. In several types of social insect, the mothers live a long time and a negative correlation has been observed between maternal age and fertility (Giron and Casas 2003; McIntyre and Gooding 2000. Information on the impact of maternal age on offspring in such long-lived social Hymenoptera is however lacking, which is surprising in view of the economic relevance of these insects.

Because, in social insects, the queens mate only at the beginning at their lives, the stored semen ages as the mother ages. Such storage is a wide-spread phenomenon, with sperm being stored naturally after mating for months or even years in a specific female organ in several species of insects, birds and reptiles, e.g. grasshopper

Chorthippus parallelus Zett (Reinhardt *et al*. 1999), chicken (Nalbandov and Card, 1943), European hare *Lepus europaeus* (Stavy and Terkel 1992), horse *Equus caballus* (Day 1942), garter snake *Thamnophis sirtalis* (Rahn 1940; Halpert *et al*. 1982; Birkhead 1993) and turtles (Gist and Congdon 1998; Pearse *et al*. 2001). Among these, honey bee queens appear to be an ideal model organism for studying sperm storage, because honey bee semen survives for several years (Locke and and Peng, 1993; Winston, 1987) and this species can be easily managed in apicultural research. Young honey bee queens mate during their nuptial flight with a number of drones (Lobo and Kerr, 1993). The semen is stored in the spermatheca of the queens for the duration of their lives and is kept alive and capable of fertilisation for up to five years (Taber, 1954; Verma, 1974; Weirich *et al*., 2002).

The honey bee is a social insect with one of the most complex social behaviours within the bee world. It is considered to be one of the most important insects on the planet. Nearly a third of our daily diet has been estimated to come from crops pollinated by honey bees (Williams 1994; Gallai *et al*. 2008). Because of the importance of honey bees to agriculture, investigations of the factors affecting the longevity and motility of spermatozoa within the spermatheca of the honey bee queen and paternal fertility are of great interest. Little information about the mode of sperm conservation within the spermatheca of insects, including honey bee queens, is however available. No previous reports exist on the importance of queen or semen age on honey bee offspring and such results could have significance for beekeeping in terms of queen performance and colony production.

1.2 Aims of the thesis:

The present study aimed to quantify the effect of parental and semen age on the longevity and health of offspring. Our specific objectives were

(1) to study the characteristics of the spermathecal content of old and young honey bee queens,

(2) to evaluate maternal age effects on the embryo mortality and juvenile development of the offspring in the honey bee, and

(3) to investigate the early embryonic mortality and embryo quality in the offspring derived from semen of different ages

2 Experimental part of the study

2.1 Impact of ageing on the quality of honey bee semen

2.1.1 Literature Review

Several studies have shown a decrease in sperm quality during storage in various species (Reinhardt *et al*., 1999; Nalbandov and Card, 1943; Stavy and Terkel, 1992; Day, 1942; Rahn, 1940; Halpert *et al.*, 1982; Birkhead, 1993; Gist and Congdon, 1998; Pearse *et al*., 2001); sperm can be stored naturally after mating for months or even years in a specific organ found in the mated females. However, little information is available regarding sperm conservation within the spermatheca of insects, including those of honey bee queens, which mate early in life during the nuptial flight with a number of drones (Lobo and Kerr, 1993). The stored semen is capable of fertilisation for up to five years (Taber, 1954; Verma, 1974; Weirich *et al*., 2002). The total number of spermatozoa extracted from the spermatheca of freshly mated queens varies between 1 and 8 million per queen (Königer and Königer, 2000; Cobey, 2007). .

Sperm have been shown to retain their respiratory activity during storage but are presumably therefore at high risk of oxidative damage (Weirich *et al*., 2002; Collins, 2004). Weirich *et al*. (2002) have established that extracts of the spermatheca of mated queens show remarkably high activities for the following enzymes: catalase (CAT), glutathione S-transferase (GST) and superoxide dismutase (SOD). They conclude that these spermathecal enzymes contribute to the protection of the spermatozoa from oxidative stress and thereby facilitate their long-term survival. These enzymes can increase sperm longevity by reducing the levels of damaging reactive oxygen species (ROS; hydroxyl, hydroperoxyl radicals and hydrogen peroxide) (Pardini, 1995). In agreement, Collins have found catalase transcripts particularly in the reproductive tissues and semen of the male and female honey bee, thereby providing further evidence of antioxidative protection. Kraft *et al*. (1978) have performed a comparative analysis on sperm CO_2 production and found that the

respiratory glucose consumption of honey bee spermatozoa is relatively low compared with the sperm of other species.

2.1.2 Results

Manuscript 1

Characteristics of the spermathecal contents of old and young honey bee queens

H. Al-Lawati, G. Kamp, K. Bienefeld

Journal of Insect Physiology (2009), 55:116-121

Summary

Naturally mated queens of known age were sampled from several beekeepers and different locations and kept in small mating bee nucs until the time of the experiments. In two sets of experiments during 2005 and 2006, semen from the spermatheca of each of 14 freshly mated (Y0), 14 one-year-old (Y1) and 7 two-year-old (Y2) queens was analysed. In addition in 2006, the contents of the spermatheca of each of 20 one-week-old virgin queens and 20 one-month-old virgin queens and fresh semen (36 µl) collected from 46 mature drones were examined. Semen of drones was collected after Mackensen and Roberts (1948). Semen of drones and the spermatheca of unmated queens were investigated to determine whether the enzyme activities measured in spermathecal extracts of mated queens derived from the contained sperm or from the spermathecal tissue**.** Using fine point forceps, the spermatheca was removed from each queen, placed in a 1.5 ml micro-centrifuge tube and disrupted by sonication. The content of each spermatheca (not exceeding 1 µl) was compared with fresh semen samples (on average 4 µl each). The sperm movement patterns were observed in 2.3 µl sperm suspensions in 20 micron analysis chamber slides (Leja, 2153 GN Nieuw-Vennep, The Netherlands) and analysed by a computer-assisted semen analysis (CASA) system (ESHRE Andrology Special Interest Group, 1998; Kime *et al*., 1996). Multiple photomicrographic exposure and video-micrographic techniques for spermatozoa track analysis (Boyer *et al*., 1989) and a

computer equipped with imaging software (Adobe Photoshop and Quik Time) were used to measure the speed of individual sperm. Five randomly selected sperm per queen were analysed. Speed was calculated (in micrometers per second) by noting the first location of the head and the second location of the head. The number of spermatozoa per spermatheca was counted by using a standard hemacytometer counter chamber slide under a light microscope (Shiran *et al*., 1995; Lu *et al*., 2007). Maximum activities of lactate dehydrogenase (LDH, EC 1.1.1.27), glyceraldehyde 3-phosphate dehydrogenase (GAPDH, EC 1.2.1.12) and arginine kinase (ArgK, EC 2.7.3.3) were spectrophotometrically analysed by using NAD(P)H as an indicator at 340 nm according to Bergmeyer (1983). The sperm from the spermatheca of older queens moved more slowly ($F = 11.45$, $P < 0.0001$) and showed different movement patterns (in straight lines rather than in circles; $Chi^2 = 90.0$, $P < 0.0001$) from those of the other groups. The spermatheca content of differently aged mated queens differed significantly with respect to the activities of lactate dehydrogenase ($F = 3.37$, $P < 0.05$), citrate synthase ($F = 6.24$, $P < 0.005$) and arginine kinase ($F = 9.44$, $P < 0.0006$). Glyceraldehyde 3-phosphate dehydrogenase ($F = 0.10$, $P = 0.91$) did not differ significantly. The results suggest considerable changes in the energy metabolic profile of the spermatheca tissue, of the sperm or of both during sperm storage.

2.2 Impact of age of mother on EEM and embryo development in then honey bee

2.2.1 Literature Review

The impact of maternal age on offspring has been reported in many species (Parsons 1964, Mousseau and Dingle 1991). Maternal age has been found to affect the viability of larva in *Drosophila* (Hercus and Hoffman 2000, Kern *et al*., 2001, Priest *et al*., 2002), hatchability in beetles (Fox 1993), growth and immune reaction in birds (Saino *et al*., 2002) and lifespan in many species (Lansing 1947, Ludwig and Fiore 1960, Raychaudhuri and Butz 1965, Fox *et al*., 2003, Priest *et al*. 2002).

Maternal age effects are assumed to affect directly not only the life history traits of offspring (Hercus and Hoffmann 2000), but also the evolution of ageing (Priest *et al*.,

2002). In social insects, especially honey bees, a wealth of literature exists on the evolution of social behaviour (Crozier and Pamilo 1996) and senescence (Remolina *et al*., 2007 and references therein) but information on the impact of maternal age on offspring characters is lacking. Flanders (1959) has suggested that the embryonic starvation found in some colonies might be exacerbated by the ageing of the queen.

Akyol *et al*. (2007) have found declining brood production and less honey in colonies as queens age, possibly accompanied by higher mortality and inferior quality in their offspring. The comparatively poor knowledge of maternal age in social Hymenoptera is surprising, because maternal effects have long-term and complex influences on progeny. In this study, we have used the ability of readily observing embryonic development within the egg and later larval development for gaining insights into the impact of maternal age on early embryonic mortality and on the ontogenesis of offspring in this long-lived social species.

2.2.2 Results

Manuscript 2

Maternal Age Effects on Embryo Mortality and Juvenile Development of Offspring in the Honey Bee (*Hymenoptera*: *Apidae*)

H. Al-Lawati and K. Bienefeld: Ann. Entomol. Soc. Am 2009, 102 (5): 881-888 2009

Summary

A total of 76 queens were categorised into 3 age groups: freshly mated (Y0), *n* = 29; 1-year-old (Y1), *n* = 29; and 2-year-old (Y2), *n* = 18. Y1 and Y2 queens were gathered from several beekeepers at different locations in Germany. The queens were introduced into mating nuclei (F. Wienhold Lauterbach, Germany) to adjust for any potentially different capacities in egg laying of the differently age queens. The nuclei contained 2 combs with nectar and pollen, and 3 brood combs and ca. 3000 bees of mixed and unrelated origin. Only queens producing completely diploid broods were used. The ages of the collected queens were indicated with numbered disks or by colour coding according to the International Queen Marking Colour Code. In

addition, virgin queens of a different origin (Y0) were reared and allowed to mate naturally. For the measurement of early embryonic mortality (EEM), in 2005 and 2006, empty combs were introduced into the mating nuclei, and eggs that were laid within a 6-hr interval were used for testing embryo mortality. Batches of 20 eggs each from 45 queens (Y0 = 17, Y1 = 17, Y2 = 11) were investigated. The egg area at the beginning of the embryonic development (8h ± 2) was measured to obtain an estimate of the amount of nutrition provided. Eggs were incubated inside a Petri-dish with wet filter paper (34.5°C, 75% relative humidity) for further embryonic observations. Embryonic mortality was observed by covering each egg with paraffin oil in order to make the chorion transparent (Counce 1961) and to allow *in-vivo* observation of the embryo within the eggs (DuPraw 1967, Moritz 1997). Any damaged eggs were excluded. For measurements of embryonic development (experiments conducted in 2006), a total of 310 freshly laid eggs (8hrs ± 2), i.e. ten eggs from each queen (Y0 = 12, Y1 = 12, Y2 = 7), were investigated for embryonic development, which was measured at 8-hr intervals after laying. The length of the embryos was measured as the distance between the anterior and posterior pole of the egg chorion, neglecting the amnion-seroza (Milne *et al*. 1988). The width was measured in the middle of the long axis of the egg. Embryos that did not develop completely were excluded from analysis. The area of the eggs was measured on the outer shell of the egg. The ratio of length/width was used in embryos and larvae as an indication of possible non-proportional development.

The offspring of 25 queens were tested for larval development (Y0 = 8, Y1 = 12, Y2 = 5). Ten larvae per queen were collected after 6-12 hrs and reared artificially (34.5°C, 75% relative humidity). Rearing under standardised *in vitro* conditions was used to compensate for the foster effect of colonies headed by differently aged queens. For the rearing of worker larvae, we used the diet described by Genersch et al. (2005), consisting of 66% royal jelly (v/v), 3% glucose (w/v) and 3% fructose (w/v) in sterile distilled water. The food was stored at 4°C and pre-warmed to 35°C before being fed to the larvae. We measured the larvae at days 1, 3 and 5. Larval length was measured from the anterior to posterior pole, and the width was measured at the middle of the larval abdomen.

Micrographs of embryos and larvae at the described intervals were taken with a digital camera (Moticam 2300) connected to a light microscope. Length, width and volume were measured by means of special photo software (Motic Images Advanced 3.2).

Statistical analysis (by SAS 2003) considered the age group and the mother nested within the age group to obtain LS means (± SE) for the maternal age effect adjusted for individual mother effect. The Tukey test was applied for multiple comparisons of means. Values were considered to be significant at $P < 0.05$. Chi^2 analysis was performed to test the differences in mortality rates in the offspring of differently aged queens.

Embryo mortality increased significantly with maternal age (Y0 = 9.12%, Y1 = 13.54% and Y2 = 30.73%; $Chi^2 = 44.65$, $P < 0.0001$). Egg size declined significantly ($F = 36.04$, $P = 0.0001$) with queen age, did not affect embryo mortality, but did influence embryo size within the egg. (r = 0.54 to 0.95). Embryo size until hatching, observed under standardised *in-vitro* conditions, was highly significantly affected by the mother's age. Maternal age significantly influenced larval size at an early stage (day 1), but not at later larval growth. Compensatory growth and non-random sampling attributable to higher mortality, especially in Y2 offspring, might explain the smaller impact of maternal age in the later larval stage. Embryo mortality was extremely high (55.7%) in the offspring of Y2 queen during the experiments on embryo growth. This indicates that juvenile stages of older mothers are much more sensitive to stress than the offspring of younger mothers.

2.3 Impact of the age of the semen on EEM and embryo development in then honey bee

2.3.1 Literature Review

Maternal age influences the offspring quality of many species such as birds (Ahn *et al*., 1997; Dzialowski and Sotherland, 2004) and insects (Parsons, 1964; Mousseau and Dingle, 1991). Significantly less is known about the impact of paternal age on

fertility and offspring quality (Nikola *et al*. 2004; Bogdanova *et a.l* 2006; Charpentier *et al.* 2008). Even fewer results are available on the duration of storage of semen on the quality of offspring. Salisbury and Flerchinger (1967) have reported that the age of bull sperm (aged from day 1 to day 5 after collection) influence embryo survival in the cow. Similar results have been observed for the guinea pig (Young 1931; Soderwall and Young, 1940). In the rat, prolongation of residence of spermatozoa in the female genital tract for abnormal intervals prior to fertilisation results in the failure of spermatozoa to fertilise all of the ovulated eggs (Soderwall and Blandau 1941). Sperm stored for 48 and 72 hrs within the genital tract of female birds tend to give abnormal embryos (Nalbandov and Card, 1943; Dharmarajan, 1950). Pingel *et al.* (1985) have noted higher mortality in offspring originating from semen stored for long periods inside hens. Similar results have been reported for swine spermatozoa stored for 6 or 54 hrs after collection (First *et al.,* 1963), with the aged spermatozoa initiating fertility but causing increased embryonic or early fetal loss. Miller and Blackshaw (1968) have reported decreased fertility in the rabbit, and increased embryo loss has been observed in the sea urchin after sperm storage (Dungay 1913; Medes 1917). In an investigation of fresh frog eggs fertilised by spermatozoa aged for 24 ± 1 hrs, the number of embryos that hatched is significantly lower than that after fertilisation with fresh sperm (Hart and Salisbury, 1967).

Most of these species do not store semen naturally for long periods of time. However, honey bee queens are able to store semen in their spermatheca for up to 5 years (Taber, 1954; Verma, 1974; Weirich *et al.,* 2002). To date, no literature is available regarding the impact of such long-term natural storage on the mortality and growth of offspring.

2.3.2 Results

Manuscript 3

Early Embryonic Mortality (EEM) and Embryo Quality in Offspring of Young Honey Bee Queens Inseminated with Semen from the Spermatheca of Freshly Mated and Two-Year-Old Queens.

H. Al-Lawati and K. Bienefeld (In preparation)

Summary

The spermathecae of 60 two-year-old and 40 freshly mated honey bee queens were sampled in the summer of 2007 and the semen was extracted from the spermatheca by using an artificial insemination needle. On average, the content of five spermathecas were used for re-inseminating one virgin queen. Eight virgin sister queens were inseminated with the semen of the spermatheca of the two-year-old-queens (2YS). As controls, six virgin queens of similar descent were inseminated with the semen from the spermathecae of freshly mated queens (0YS). This experimental design allows for adjustments to be made for maternal age and the effect of semen manipulation for the rearing of queens and artificial insemination, the standard protocol of Ruttner (1976) was used. We tested for successful insemination by inspecting the first set of the sealed brood for the incidence of worker brood (i.e. fertilised eggs). In addition, a random sample of 10 embryos per queen was tested for ploidy by using polymorphic DNA 4 micro-satellite markers (Etoup *et al.,* 1995) in order to check whether unfertilised eggs (which develop into drones and may have a different mortality rate) occurred. All embryos tested were found to be heterozygotes and therefore females.

The queens were kept in mating nuclei (F. Wienhold, Lauterbach, Germany), which contained 2 combs with nectar and pollen, plus 3 brood combs and ca. 3000 bees of mixed and unrelated origin.

From each queen, 40 eggs were sampled at the age of 6 hrs ± 2. Egg age was standardised by inserting a new empty comb and allowing the queen to lay eggs on it. The eggs were transferred onto a microscope slide and totally covered with

paraffin oil in order to make the chorion transparent. From each queen, 10 freshly laid eggs (6 hrs ±2) were used for observing embryo development. To obtain eggs at a standardised age, a new comb was introduced into the nucs for 6 hrs. As for embryonic mortality, development was observed after covering the eggs with paraffin oil and incubating them at 34.5°C and 75% relative humidity. Embryonic development was measured every 8 hrs after egg laying. The length of the embryos was measured as the distance between the anterior and posterior pole of the egg chorion (Milne *et al.,* 1988). The width was measured at the middle of the long axis of the egg. Embryos that did not develop completely were excluded from the analysis. The area of the eggs was measured on the outer shell of the egg. The ratio of length/width (L/W) was used in embryos and larvae as an indicator for possible non-proportional development.

Offspring of the 14 queens were collected at 6-12 hrs and reared artificially (34.5°C; 75% relative humidity). Rearing under standardised *in vitro* conditions was used to compensate for a possible foster effect of the different nuclei. For rearing worker larvae, we used the diet described by Genersch et al (2005). The larvae were measured at days 1, 3 and 5. Larval length was measured from the anterior to posterior pole, and the width was measured at the middle of the larval abdomen.

Micrographs of embryos and larvae were taken at the described embryo ages with a digital camera (Moticam 2300) attached to a light microscope. Parameters were measured by means of special photo software (Motic Images Advanced 3.2). All statistical analyses were computed by using Statistical Analysis System SAS 9.1 software package (SAS 2003). The analysis of variance (ANOVA) of egg, embryo and larva size considered the effect of semen age and the mother nested within the semen age group. Chi^2 analysis was performed to test the differences in the mortality rates of the offspring in the two semen age groups.

Embryo mortality was high in both semen age groups. The high mortality in the control (0YS) indicated that use of a semen extraction and re-insemination technique had per se an effect on embryo mortality. Nevertheless the effect was significantly higher in offspring originating from old semen (2YS: 52.3%, $Chi^2 = 9.79$, $P < 0.0018$). The mortality in the 0YS group was found to be 37.8%. Embryos originating from old semen were found to be smaller at all stages, but the effect was shown to be highly

different only at 8 hrs, 48 hrs and 72 hrs. Larval size differed only significantly at day one.

3 General Discussion

Sperm quality decreases during storage in various species (Reinhardt *et al*., 1999; Nalbandov and Card, 1943; Stavy and Terkel, 1992; Day, 1942; Rahn, 1940; Halpert *et al*., 1982; Birkhead, 1993; Gist and Congdon, 1998; Pearse *et al*., 2001). Sperm obtained from the spermatheca of young queens (Y0) perform exclusively circular movements, whereas those from older queens tend to move more slowly and in straight lines. Circular movement has also been found in the sperm of the rove beetle (*Aleochara bilineata*) by Werner et al. (1999) who have assumed that this kind of movement prevents entanglement of the long spermatozoa during storage within the spermatheca. This may also help individual spermatozoa to leave the mass of sperm for fertilisation. Lefevre and Jonsson (1962) have observed the continual circulation of spermatozoa in the receptacle of *Drosophila melanogaster* and suggest that this motion helps to release the sperm individually. Although these species differ with respect to length of semen storage, the mechanism may be similar between species. The packaging of sperm at high density is probably one of the general strategies for long-term storage, whereas the circular movement, as seen here in the honey bee, is a pre-requisite for the release of single spermatozoa from the spermatheca.

Hypertonicity within the spermatheca is probably the second strategy partly responsible for the successful and long-term storage of spermatozoa in the queen honey bee (Verma and Shuel, 1973; Verma, 1974). The osmotic pressure of the spermathecal fluid has not been measured directly in the experiments reported by the Verma group, but the calculated value (based on the total ionic and sugar concentrations measured in the spermatheca) is as high as that of whole semen, seminal plasma or queen haemolymph (Verma, 1974). This hypertonicity probably keeps metabolism low during storage and results in the circular movement of the spermatozoa.

As the axoneme is anchored to the base of the sperm head, any sliding force is translated into a bend in the flagellum. Since dynein produces force in only a single direction (Sale and Satir, 1977), the generation of a normal flagellar waveform requires that phosphorylation/dephosphorylation and the associated activation and inactivation of the dynein arms occur in an asynchronous manner around the

circumference and along the length of the axoneme. Thus, the circular movement of the sperm, as seen in the sperm obtained from the spermatheca of young queens, is associated with a minimum or negligible flagellar beat frequency, requiring low energy output (Cardullo and Baltz, 1991). Forward motion (higher beat frequency), as seen in the sperm from the older queens, might be more expensive and hence the sperm from these queens tend to move more slowly or are even immobile.

The sperm motility of many species is tightly coupled to ATP production by mitochondria, because the complete oxidation of substrates such as sugar, fat or proteins requires mitochondria and the ATP yield is much higher (36 ATP/glucose) than that of the mitochondria-independent fermentation of sugar (2 ATP/glucose) to lactate. In the spermatozoa of many species, mitochondria are localised in a short piece of the flagellum and therefore the energy has to be transported to all the dynein-ATPases along the flagellum. Studies of sea urchin sperm have lead to the hypothesis of an energy transport system (Tombes and Shapiro, 1985, 1989); the energy-rich phosphate of ATP is transferred to creatine by mitochondrial creatine kinase and the product phosphocreatine is transported to the dynein-ATPases. This phosphocreatine/creatine kinase system has been found in the sperm of many species of different phyla, whereas spermatozoa of arthropods and molluscs possess the phosphoarginine/ArgK system (Watts, 1971). In agreement, ArgK has been found in bee spermatozoa and its activity is relatively high. This result seems to be of general interest, because the mitochondria in bee spermatozoa are not restricted to the short piece of the flagellum. The tails contain two mitochondrial strands that extend from the base of the nucleus nearly to the end of the tail (Rothschild, 1955). Therefore, the question arises as to whether a phosphoarginine/ArgK shuttle is necessary. Further analysis concerning the role of ArgK and its compartmentation in bee sperm should be carried out.

Research on sperm energy metabolism in the honey bee is limited (Collins *et al.* 2006). Sperm energy metabolism can be characterised by the maximum activities of marker enzymes reflecting the capacities of the various metabolic pathways. The presence of glyceraldehyde 3-phosphate dehydrogenase (GAPDH) indicates a capacity for glycolysis, whereas lactate dehydrogenase (LDH) activity shows the extent to which lactate production or consumption occurs. Citrate synthase (CS) is a

marker of the citric acid cycle, and arginine kinase (ArgK) reflects the potential of phosphagen turnover and of the proposed phosphagen shuttle in the spermatozoa (Strong and Ellington, 1993; Kamp *et al*., 1996; Kaldis *et al*., 1996). Our results agree with those of Blum and Taber (1965) who have found activities for various dehydrogenases associated with both the Krebs cycle and glycolysis in honey bee spermatozoa and contradicts Kraft *et al*. (1978) who report that honey bee sperm do not use the Krebs cycle. Whereas we have found that all enzymes are present in spermatozoa obtained from fresh semen of drones, LDH and GAPDH seem, in particular, to be synthesised in the spermathecal tissue of queens within one month after hatching. The activities of all the studied enzymes are fairly constant in queens within one year after mating indicating good conservation of the capacity to produce ATP. After two years, however, the activities of the mitochondrial enzyme CS and ArgK decrease more than those of LDH and GAPDH. Whether this is an effect of mitochondrial damage has still to be investigated.

We have also determined the effect of the use of stored (aged) sperm on the viability of offspring (see section 2.3). This is the first observation of early embryonic mortality and embryo development following fertilisation with semen naturally stored for 2 years and adjustment for maternal age. Old sperm is still fertile but has a significant deleterious effect on embryo development. DNA may deteriorate and thus sperm may accumulate genetic damage as they age (Siva-Jothy 2000). Thus, the ageing of sperm may explain why the egg hatching frequency and offspring egg-to-adult survival both decrease. A large semen age effect has been detected on embryonic mortality during early development but this effect decreases as the offspring become older. No significant influence of semen age is detectable on the body size in larvae older than one day, possibly because of compensatory mechanisms.

Embryo mortality is strongly affected by the age of the mother in the honeybee. The offspring of 2-year-old queens show a more than three time higher mortality than the offspring of young queens. A negative correlation between maternal age and hatching rate of offspring has also been shown by Fox (1993), Hercus and Hoffman (2000) and Kern *et al*. (2001). The smaller size of eggs laid by older females is assumed to affect the hatching rate (Azevedo *et al*. 1997). As in our study, egg size has generally been reported to decrease with maternal age (Wiklund and Persson

1983, Jones *et al*. 1982, Fox 1993, Mohaghegh *et al*. 1998). This decrease in egg size is generally attributed to a depletion of the female's resources, such that later-laid eggs are smaller. Although eggs from old queens are on average 30% smaller than those from young queens, we have not found an association between egg size and embryo mortality. Egg size in a large range of taxa has been shown to be positively correlated with survival (Gall 1974, Wallace and Aasjord 1984, Marshall and Bolton 2007). However, several studies support our results of a lack of association between egg size and embryo mortality (Williams *et al*. 1993, Meathrel *et al*. 1993, Jónsson and Svavarsson 2000). As shown by McIntyre and Gooding (2000) and Giron and Casas (2003), the maternal age effect might involve poorer egg quality, a feature that possibly also exists in the honeybee.

Giron and Casas (2003) and McIntyre and Gooding (2000) have reported a marked decrease in reproductive investment in eggs with maternal age, as regards egg size and egg content in the parasitic wasp (*Eupelmus vuilletti)* and in the house fly (*Musca domestica),* respectively. A vast body of literature exists with respect to the impact of maternal investment on the body size of the offspring of various species (Bernardo 1996 and literature therein). We have observed a highly significant decrease in egg size as queens age and a highly positive correlation between individual egg size and embryo size shortly before hatching. The large and more uniform egg size in young queens seems to provide, but only to a minor degree, a limiting factor for embryonic development. In the significantly smaller and more variable egg size in older queens, the developing embryos seem to reach the limits of maternal provision more frequently.

In addition to these quantitative and qualitative egg provisioning effects on embryo mortality, other factors are assumed to contribute to early embryonic mortality. Mitochondrial DNA is known to be the major target of oxidative damage and may, therefore, be responsible for the age-related accumulation of the genetic load (Wallace 1994). Evidence for this hypothesis has been presented by Gavrilov *et al*. (1997). An age-related accumulation of mutations may influence embryo mortality in a twofold manner: directly by mutation in the germ cells and indirectly via mutations in the somatic cells of the mother, which may additionally decrease her potential for efficient egg provisioning. Another potential mechanism involves the weakening of

the microtubule network during meiosis in ageing oocytes; this can result in a increased frequency of aneuploidy or trisomic zygotes in older mothers (Schatten *et al*. 1999).

We have detected a large maternal age effect on mortality and embryo size in early development, as shown above. However, a significant maternal age effect on larva size is only found at the beginning (1st day) of larval growth; no significant maternal age effect has been observed on larval size in later developmental phases. Because of the link between adult mass and fitness (Roff 2002), animals are expected to compensate for any stress that would negatively affect their final mass. Compensatory growth is a well-known phenomenon in all species (Wilson and Osbourn 1960). In general, maternal effects are strongest early in ontogeny and are diluted during development as the genes of the offspring are expressed (Rutledge *et al*. 1972, Lindström 1999). The finding that larvae of differently aged mothers do not differ in size at later developmental stages does not mean that they will not suffer a disadvantage in later life. Several studies in diverse taxa have shown that, even if an organism appears to recover from deprivation when resources subsequently improve, nutritional deficits during early development can have profound, pervasive and permanent effects on the adults and their offspring (Metcalfe and Monaghan 2001 and 2003, Fischer *et al*. 2006, Block *et al*. 2008). However, we assume that offspring disadvantaged from being produced from old semen (see also above) compensate for the shortage of food at the early stage of development, i.e. that, after hatching, the larvae eat more and grow faster to compensate for the disadvantages incurred during early development (Metcalfe and Monaghan 2001; 2003)

The maternal age effect found at the beginning of larva development in the honey bee is probably influenced by differences in the egg size of the differently age mothers. Nevertheless, we cannot exclude additional age-related effects, such as maternal age being more closely linked to qualitative nutrition effects (Bernardo 1996) and/or an accumulation of genetic abnormalities in eggs as mothers age (Crow 1997). In addition, when a queen ages, the semen stored in her spermatheca also ages. We have shown above (see also section 2.1) that honeybee semen in old and young spermatheca strongly differ. Results obtained by Priest *et al*. (2002), who have observed significant paternal age effects (but to a lower extent than maternal age

effects) on offspring longevity, indicate that semen age can indeed impact on offspring.

Another result of this study is worth mentioning here. We have observed extremely high mortality (56%) in the embryo experiment with queens of 2 years of age. This experiment required repeated removal of the embryos from the incubator and their exposure to the light of the microscope at room temperature. The offspring of aged queens are much more sensitive to this experimental stress than the offspring of younger mothers. We therefore generally assume a higher responsiveness of juvenile stages of older queens to several stress factors than offspring from younger mothers.

4 General Conclusion

The queen honey bee (*Apis mellifera*) stores sperm in the spermatheca for several years. Little information is however available regarding the effect of such long-term storage of sperm on their fertility. We show here that sperm from the spermatheca of older queens move more slowly and have different movement patterns from those of the younger group. This has been correlated with the considerable changes that occur in the energy metabolic profile in the spermatheca content of the differently aged mated queens.

In addition, early embryonic mortality has been found to be higher within the eggs from old queens than in eggs from younger queens. Egg volume and consequently embryo size decrease as the queen ages. Maternal age significantly influences larval size at an early stage (day 1), but not at later larval growth. Compensatory growth and non-random sampling attributable to higher mortality, especially in offspring from the older queens, might explain the smaller impact of maternal age in these later larval stages.

Because of the mating strategy, maternal age and semen age are usually confounded in species exhibiting long-term sperm storage. A new technique has been developed, which allows adjustment for different maternal ages. Embryo mortality is high in both semen age groups, but the deleterious effect is significantly higher in the offspring originating from old semen, This suggests that sperm age also impacts on the development of the offspring.

Gallai *et al*. (2008) have estimated a world value for the contribution of insect pollinators to the production of crops at € 153 billion, which is about 9.5% of the total value of the worldwide production of human food. The honeybee plays a decisive role in this production (Williams 1994). Consequently, every factor that influences pollination ability is of great interest. Akyol *et al*. (2007) have shown that older queens negatively affect colony performance. Because of the smaller size of colonies headed by older queens, their pollinating ability and consequently their ecological benefit are also likely to be reduced. Our data indicate that this is attributable not only to reduced laying capacity or reduced pheromone output of aged queens, but also to

the significantly increased mortality and possibly reduced vitality of the surviving offspring of old queen bees..

Comparable with the problems brought about by colony collapse disorder in the US, high colony losses have been observed for some time in Europe. The recordings in the course of the German Bee Mortality Monitoring Program of all possible factors concerning colony losses in 1200 colonies representatively distributed in Germany have revealed that three factors significantly affect mortality: Varroa, viruses and the age of the queen (unpublished report, but available under http://www.ag-bienenforschung.de/). The offspring of old queen bees seem also to respond in a significantly more sensitive manner to stresses that the honeybee colony naturally has to face. Although maternal age effects on offspring are found as early as at the end of queen's first year, a substantial decline in offspring viability is observable during the queen's second year. As a consequence for beekeeping, the use of older queens ($\geq$ 2 years) is likely to be associated with a decrease in colony performance and reduced resistance to stress-induced damage. The results of the present study thus lead to the recommendation of using young queens for efficient production.

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Declaration

I hereby declare that the work contained in this thesis is my own and contains the results of an original investigation, except where otherwise referenced or acknowledged. This work was carried out while I was enrolled as a student for the Doctor's degree in Agriculture Science in the Institute for Bee Research, Hohen Neuendorf, Humboldt University of Berlin, Germany. This thesis has not been previously submitted for examination at this, or any other, university.

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