Fall 2010

Sperm Use During Egg Fertilization in the Honeybee (Apis Mellifera)

Maria Rubinsky
SIT Study Abroad

Sperm use during egg fertilization in the honeybee (*Apis mellifera*)

Maria Rubinsky
November, 2010
Supervisors: Susanne Den Boer, Boris Baer, CIBER; The University of Western Australia
Perth, Western Australia

Academic Director: Tony Cummings
Home Institution: Brown University
Major: Human Biology- Evolution, Ecosystems, and Environment

Submitted in partial fulfillment of the requirements for Australia: Rainforest, Reef, and Cultural Ecology, SIT Study Abroad, Fall 2010

Abstract

A technique to quantify sperm use in honeybee queens (*Apis mellifera*) was developed and used to analyze the number of sperm used in different groups of honeybee queens. To do this a queen was placed on a frame with worker cells containing no eggs, and an excluder box was placed around her. The frame was put back into the colony and removed after two and a half hours. This method reduced stress on the queen so that she felt comfortable enough to lay eggs and did not require the queen to be killed so that she could be sampled multiple times to look at effects of age and time after introduction into a colony. The eggs were guaranteed to be fresh and sperm number present on the egg could be counted with a fluorescence microscope by using DAPI to dye the DNA in the sperm heads. The queens were found to be very economic in their sperm use and an overall median of three sperm per egg was found. Individual queens were found to vary significantly in sperm used per egg, however no queen had a median of over ten sperm per egg. Days after introduction into the colony also had a significant effect on sperm use when looking at the queens individually and it was found that newly introduced queens used more sperm than established queens. Overall, the study shows that freshly laid eggs can be collected and sperm on these eggs can be counted directly. This is an important finding for further research into the factors affecting different patterns of sperm use and possibly for beekeepers to breed queens who use their sperm most efficiently, and who will therefore be able to maintain a successful colony for the longest time.

Table of Contents

Acknowledgements

I would like to thank Susanne Den Boer very much for being my supervisor, helping me to set up my experiment, and helping me to analyze my data. Without her guidance, the project would have not have gone so smoothly and gotten as far as it did in such a short time. I would like to thank Boris Baer for making arrangements for me to come to the university and for his help and supervision of the project. I would really like to thank Tifane Bates for her help and support throughout the project, for giving me a place to stay, for sharing all of her knowledge about honeybees and beekeeping with me, and for taking me on some very nice adventures through the Western Australian bush. Thanks to the whole CIBER team for sharing their knowledge and suggestions with me and guiding me when I was lost.

Thank you to Tony Cummings for making this all possible and for helping me to find the perfect project. Lastly, I would like to thank Sydney Jaimes for her inspiration, without which I probably would not have ended up in beautiful Western Australia.

Introduction

Background

With the exception of mammals, most species in the animal kingdom with internal fertilization have a specialized organ that is used to store sperm between mating and egg fertilization. These include species of mollusks, annelids, arachnids, insects, crustaceans, poeciliid fish, amphibians, reptiles, birds, and bats (Pitnick et al. 1999). The spermatheca is the organ that stores sperm in some vertebrates and most invertebrates, including insects. It varies greatly in shape and histology between groups, however it usually consists of a spermathecal duct that leads from the vagina to the spermathecal bulb, and has a distinct pumping region (Kocorek and Danielczok-Demska 2002). How much sperm is stored and for how long varies considerably between the species and is highly dependent on mating frequency and number of offspring that need to be produced (Pitnick et al. 1999). For example, female snow crabs (Chionoecetes) must mate at least once every two years as their spermatheca can only hold enough sperm to fertilize two clutches of eggs, with one produced each year (Paul 1984).

As in the case of the snow crabs, most organisms can store sperm for a few months or years, but this time is usually much shorter than the lifespan of the organism. The eusocial insects in the order Hymenoptera (ants, bees, and wasps) are the exception. The queens of these colonies only mate early in their life during one occasion referred to the nuptial flight, but will never remate once they have started a colony. They use the stored sperm supply over their long lifetimes, some which can be more than a decade. For example, leaf-cutter ants (e.g. *Atta colombica*) have queens that live for ten to twenty years and have colonies with up to 8 million workers, which have all been produced with the sperm supply that was collected during a single night in the queen's life (Den Boer et al. 2009)

As in ants, honeybee (*Apis mellifera*) colonies are led by a single queen who mates only at the beginning of her life during one or very few nuptial flights. These flights usually occur in spring when males, referred to as drones, leave their nest and aggregate at specific geographic locations. The virgin queens also leave their nests and fly to these sites, where copulation occurs during flight. The entire copulation process takes only a few seconds and queens typically mate with a large number of males in quick succession. Because of the briefness of the copulation and the mass swarm of drones flying after the female, it is generally assumed to be unlikely that precopulatory mate choice is a major factor in the honeybee mating system. However, as honeybee queens acquire a lot more sperm during copulations that is actually needed for storage, queens have been hypothesized to perform cryptic female choice by rejecting or favoring some ejaculates. Males ejaculate into the bursa copulatrix and the sperm is then transferred to the lateral oviducts by contractions of the bursa (Baer 2005).

The drones die shortly after copulating because part of their endophallus breaks of in the queen after ejaculation, paralyzing them and causing them to fall to the ground. The drone leaves a mating sign consisting of several gland secretions and a chitinous plate, which may help to stop backflow of semen and guarantee transfer of the sperm to the lateral oviducts. The sperm storage process lasts until about 40 hours after mating and most sperm is lost as it flows back into the bursa copulatrix to eventually be expelled through the vagina. The lateral oviducts of a queen after a mating flight contain about 200 million sperm, but only about 4.7 million sperm get stored in the spermatheca, meaning only about 2.5% of acquired sperm are actually stored. The queen thus mates with many more males than is needed to simply fill her spermatheca and it is hypothesized that mating with multiple mates increases genetic diversity among offspring in the colony, which has many beneficial effects, including the reducing parasitism, increasing of worker task performance efficiency, and buffering colony performance against fluctuating environmental conditions (Baer 2005).

The inseminated queen returns to her hive after mating, where she is responsible for the continuous production of the workers that make up the majority of the colony, which can reach up to 100,000 individuals. On average, a colony contains 50,000 workers with a lifespan of only one month. This means that the queen must be constantly laying many eggs to maintain the colony. As in all social Hymenoptera, offspring production occurs via a haplo-diploid sex determination system, with internally fertilized eggs developing into females (either queens of sterile workers) and unfertilized eggs developing into males. A queen is capable of fertilizing millions of eggs over her lifetime and as her egg production is unlimited, it is the number of sperm stored and her efficiency of use of these sperm that determines her lifetime reproductive output. This is because when a queen runs out of stored sperm, she is only able to lay unfertilized eggs, which will develop into drones. The workers quickly detect this and kill her, as she is useless to the colony, which will not survive without the production of new workers (Winston 1991). Reproductive success is also closely associated with the number of fertilized eggs a queen can produce per time unit because larger colonies produce more sexuals and because honeybees found new colonies by colony fission. When a colony is doing well, they produce new queens and the old queen departs from the colony with a swarm of workers to found new colony. Larger swarms are more successful in establishing a new site, surviving colony initiation, hibernating, and successfully reproducing the following year (Baer 2005).

As mentioned, unfertilized eggs develop into drones, while fertilized eggs develop into workers or queens, depending on how the larvae is fed by the workers. Different cells are built by the workers for the three types of larvae: standard horizontal cells for workers, larger, raised horizontal cells for drones, and raised vertical cells for queens. The queen is extremely precise in determining whether to lay a fertilized or unfertilized egg in a cell (Ratnieks and Keller 1998). She may determine which type of egg to lay during a pre-laying inspection of the cell, in which she places her head and forelegs into the cell. Amputating part or all of her forelegs causes her to lay fertilized eggs into drone cells. She may also determine which type of egg to lay by the different abdominal angle upon ovipostion in the two different sized cells (Winston 1991).

Fertilization of the egg takes place in the vagina as the egg passes the orifice of the spermathecal duct. In honeybees, the spermatheca is a large globular sac lying over the vagina with which it is connected by the short spermathecal duct. The release of sperm is controlled by the action of muscular sperm pump of the spermathecal duct. There is a valvular fold on the floor of the vagina and when erected, it holds a passing egg that is to be fertilized by pressing against the orifice of the spermathecal duct. If the egg is not to be fertilized, the fold relaxes and the activity of the pump ceases. The pre-laying inspection and antenna stimulation also causes secretion by the spermathecal gland, which is alkaline in contrast to the weakly acidic fluid of the spermatheca. This activates the spermatozoa, which then move out of the spermatheca and into the upper part of the duct. Here the gland secretion and sperm accumulate and cause increasing pressure, which keep the valve of the duct closed. When an egg passes into the vagina, the valve opens for an instant to release a few sperm in a minute quantity of secretion (Snodgrass 1984).

Justification for Study

Because the queen's life and the success of her colony depend on her fertility, the queen must make sure to store sufficient amounts of sperm to allow fertilization of up to 1.7 million eggs throughout the rest of her life. However, storage of too much sperm might be metabolically costly to maintain and may decrease queen fitness. It has been shown that in ants, queen immune response is negatively correlated with amount of stored sperm, with queens that store more sperm investing less in an immune response to pathogens (Baer et al. 2006). Therefore, queens must store just enough sperm to last their lifetime, as storing too much might induce fitness costs. Furthermore, in the absence of any remating later in life, queens are expected to maximize the economy of sperm use during egg fertilization, to conserve as much sperm as possible while still guaranteeing fertilization.

Few studies have been done before to estimate the number of sperm used per egg and results have been widely scattered: 4-12, 10-100, 20-30, 10-12, 50-100, and 5-10 (Tschinkel 1987; Harbo 1979; Baer 2005). The variation in number may be due to the fact that these studies did not take age of the queen into account or because of the methods used. Most compared amount of sperm in the spermatheca right after mating with amount left in the spermatheca after laying certain numbers of eggs, and the number of sperm was calculated from these numbers. However, no study has directly counted the amount of sperm found on honeybee eggs and compared these between the different ages of the queens. Such work was recently done in the leafcutter ant, *Atta colombica*, and it was shown that there is a large degree of variation in sperm use between queens, with some queens using five times the number per egg than other queens. However, on average the queen only uses two to three sperm per egg and this increases with increasing queen age. This increase can be due to the fact that the queen may need to use more sperm per egg to guarantee fertilization, as sperm viability decreases with age, or because she cannot control the release of sperm as precisely as she gets older (Den Boer et al. 2009).

Aims of Study

This study aims to develop a technique in honeybees that allows quantification of sperm cells on freshly laid eggs. It also aims to get first insights into factors affecting sperm use, for example, queen age. This information will help to gain an understanding of the strategies queen's use to economize sperm use.

Methods

Apis mellifera queens were obtained from colonies present at the University of Western Australia in Perth in November 2010.

Egg Collection

Freshly laid eggs (less than two and a half hours old) were collected from seven different queens. A frame with empty worker cells was chosen and checked carefully to make sure that there were not any previously laid eggs on it. The queen was then collected and carefully placed on the chosen frame. An excluder box measuring 12.4 centimeters by 12.2 centimeters (covering 22 by 25 or 550 cells) was placed on top of her and any surrounding workers (see Figure 1). This forced the queen to stay in the designated area, but allowed the smaller workers to move in and out so that stress was reduced and feeding of the queen was ensured. The frame was then placed back in the colony and the hive closed.

Figure 1: Excluder box containing the resident queen and surrounding workers on a frame with no eggs.

After the queen had been in the excluder box for two and a half hours, the frame and excluder box were removed, the queen released back in the colony, and remaining workers brushed off into the colony. All eggs laid in the boxed area were then collected and counted using watchmaker forceps and placed individually on a glass microscope slide. When the frame was not being used, it was placed on the edge of the hive with an excluder frame between it and the rest of the hive so that the queen could not reach the frame and lay eggs on it between trials. To test for differences in sperm use between queens of different ages, four young queens and three older queens were used: queens 7, 9, and 10 were less than or equal to six months old, queen 18 was one month, queen 17 was 22 months, queen 16 was 18 months, and queen 15 was 24 months of age.

Measurement of Sperm Use

Sperm use was defined as the number of sperm found on the surface or in the cytoplasm of freshly laid eggs, assuming that all sperm released by the queen adhered to the egg. The freshly collected eggs were examined for damage and only undamaged ones were placed individually on microscope slides. They were then stained with six microliters of DAPI (4',6-diamidino-2-phenylindole) working solution. Working solution consisted of two microliters DAPI stock solution (2 mg DAPI in 1 ml dimethylsulfoxide) in 1 ml 0.1M $NaPO_4$ buffer with a pH of 7.0. DAPI is a fluorescent dye that binds to DNA and lights up when examined under a fluorescence microscope; it therefore allows the visualization of sperm heads (the only part of the sperm cells containing DNA) and egg nuclei. Cover slides were placed on the eggs causing them to burst so that sperm on the surface of the egg, as well as sperm that had already entered the egg at that time, would become stained. Eggs were examined with a fluorescence microscope and number of sperm on the chorion and in the cytoplasm was counted (see Figure 2). Between 13 and 66 eggs were counted per queen, over multiple occasions. One sperm was added to the count for each egg because it was assumed that at the time of collection a sperm had already entered and fused with the nucleus of the egg, as this process takes only 15 minutes (Yu and Omholt 1999; see also Den Boer 2009). This assumption is supported by the fact that queens are extremely accurate in laying the intended type of egg; they almost always lay fertilized eggs in worker cells and unfertilized eggs in the bigger drone cells (Ratnieks and Keller 1998).

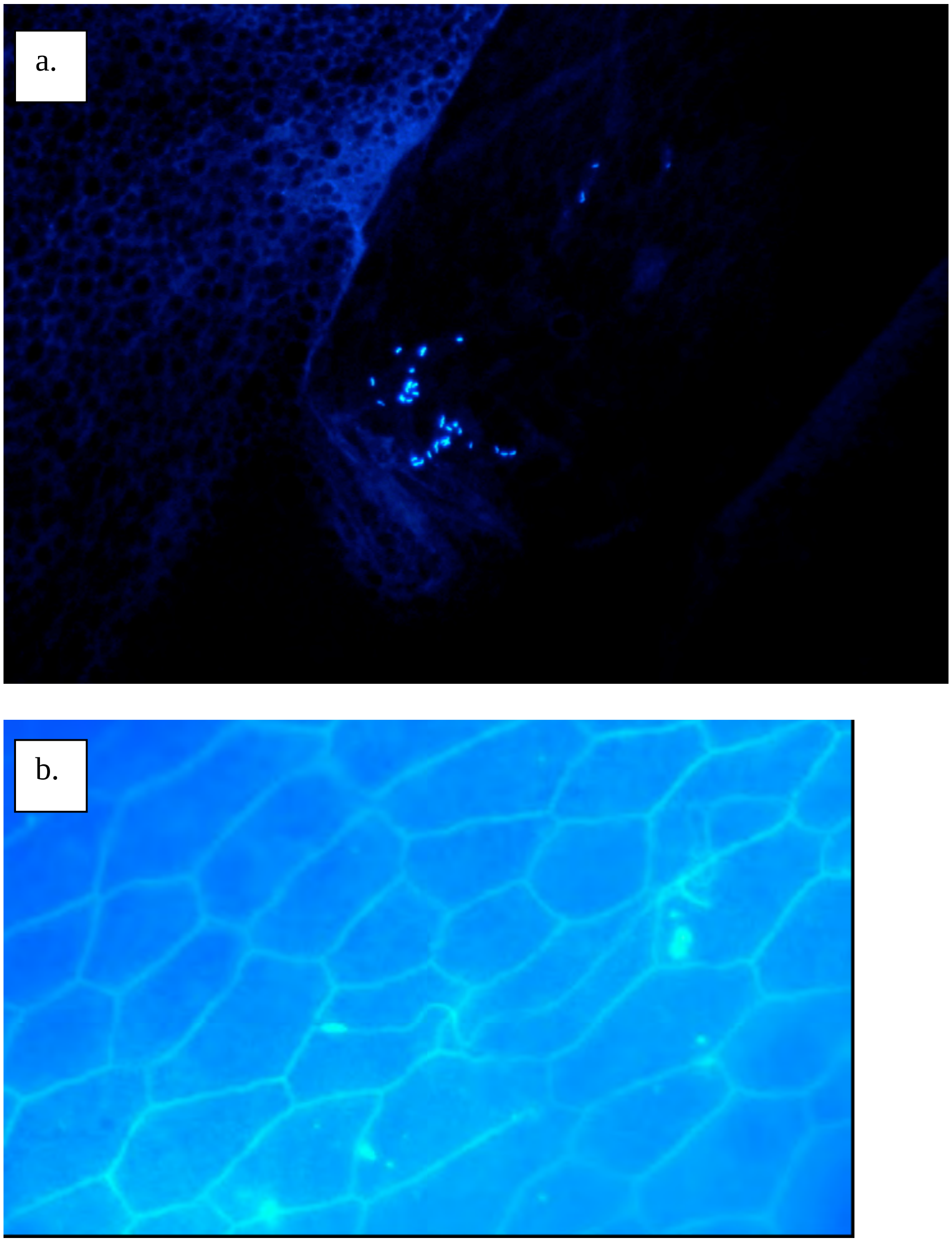

Figure 2: Sperm on the surface of a honeybee egg. Sperm was sometimes found in large groups (a). While the sperm head lit up most brightly, the tail was sometimes visible under high magnification (b). Photo by Rodolpho Jaffe.

Data Analysis

Data were analyzed using the statistical software package SPSS Statistics 19 for Windows. The distribution of sperm use was highly skewed to the right and could not be normalized through transformation for parametric testing. Therefore, a Kruskal-Wallis test was used to test for differences in sperm use across queens. For subsequent analyses for the effects of variables on sperm use, the median number of sperm per egg per queen was used.

Results

Overall, queens seemed to be very economic in using their sperm supply to fertilize eggs; an overall median of only three sperm per egg was found. None of the queens had a median over ten sperm per egg, however individual queens varied significantly in their sperm use (Kruskal-Wallis test, $H = 93.873$, $df = 6$, $p < 0.001$) (see Figure 3). For example, queen 9, 10, and 17 used a median of two sperm per egg, while queen 18 used a median of nine sperm per egg.

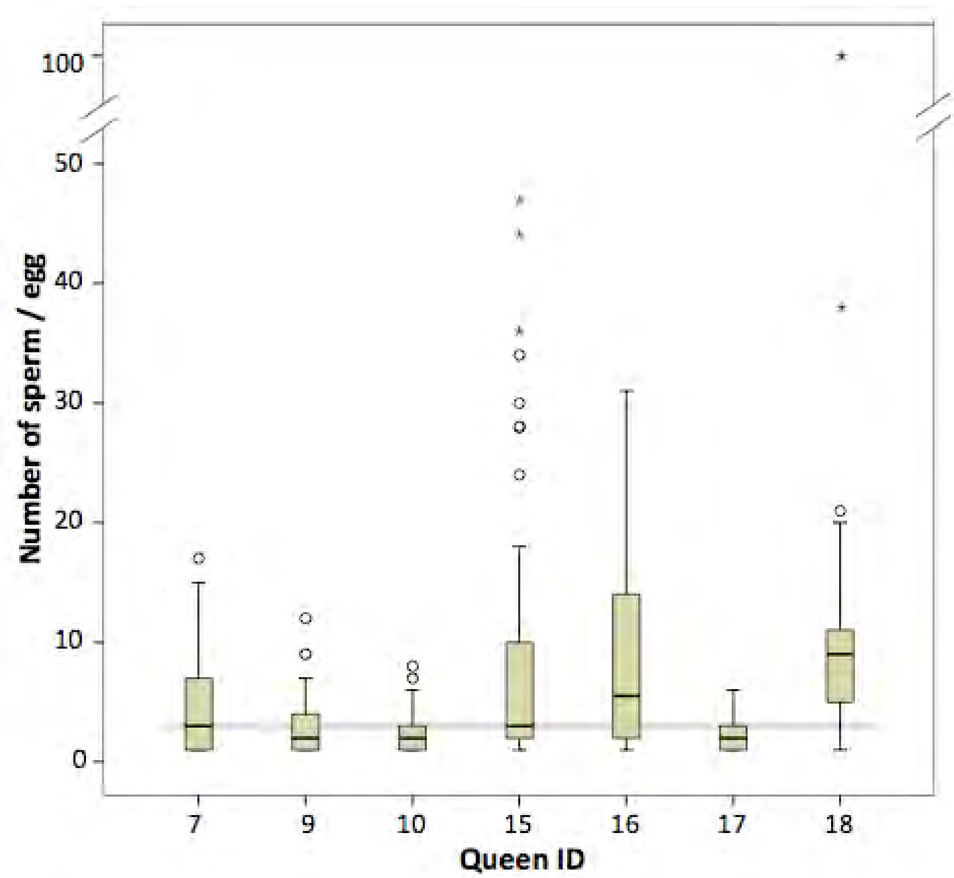

Figure 3: Boxplots showing the number of sperm used per egg by the seven different queens. Grey line depicts the overall median. The green boxes denote values between the 25th and 75th percentile and the black line inside is the median. The opens circles represent outliers, while the stars represent extreme outliers.

Queen age did not affect sperm use (r= 0.334, n= 7, p= 0.464) (see Figure 4); both the young (≤ 6 months) and old (≥ 18 months) queen groups had a median of three sperm per egg.

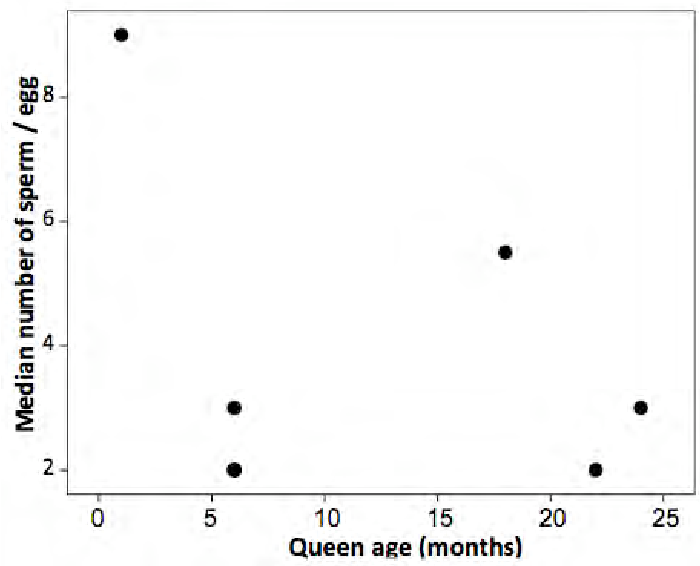

Figure 4: Median number of sperm per egg in relation to queen age in months. Each point represents the median sperm use of a single queen.

Whether sperm use was affected by how long queens had resided in the colonies when their eggs were collected was also examined to see if the queens signal their fertility to prevent rejection by the colony when first introduced (beekeepers frequently relocate queens to a new colony with unrelated workers). Time after introduction into a colony did not seem to seem to affect sperm use patterns (r= 0.562, n= 7, p= 0.190), however the interaction factor queen*time after introduction was significant (ANCOVA; F = 7.125, df = 6, p = 0.007), indicating that the 7 queens differently adjust their sperm use patterns with time after introduction. This is illustrated in Figure 5; Queen 15, 16 and 7 decrease in the number of sperm they use per egg over time, while queen 17, 18, 9 and 10 stay around the same median. This graph furthermore shows that there is a clear difference in overall medians between the first cluster of data points (<11 days after introduction, overall median sperm use = 6.23 sperm per egg) and the second cluster (>

67 days after introduction, overall median sperm us = 1.68) and this difference is significant (t-test, t = -3.422, df = 20, p = 0.003).

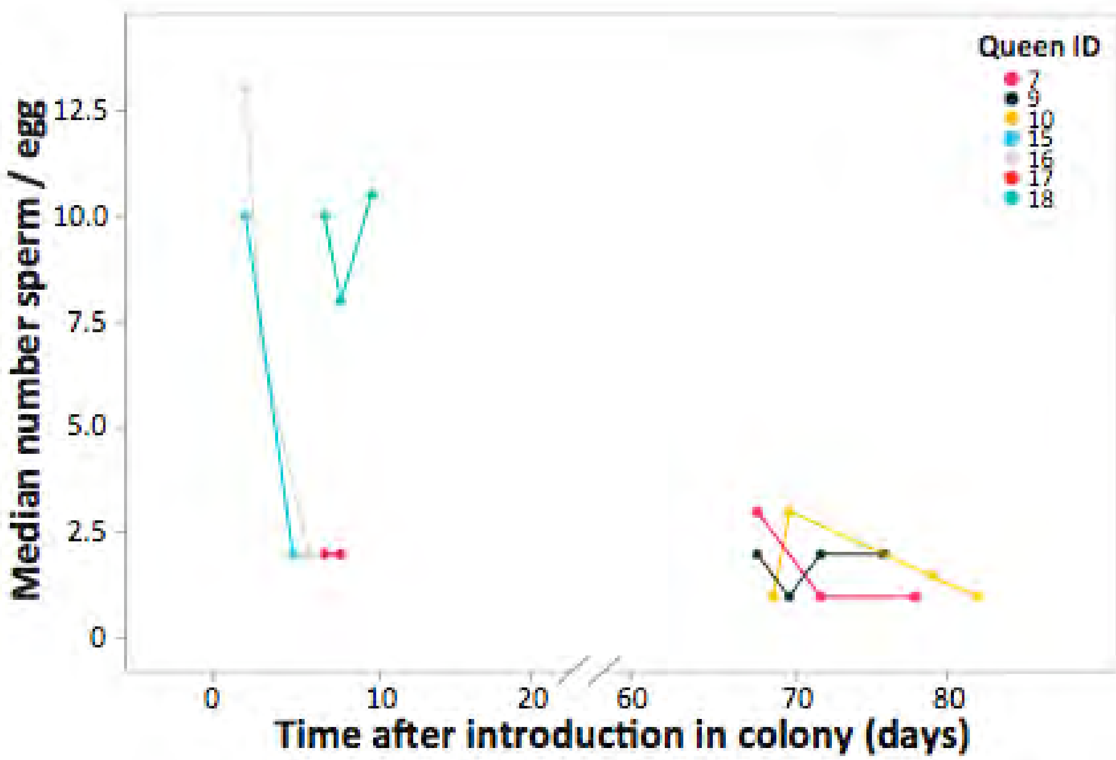

Figure 5: Median number of sperm used per egg by the 7 different queens in relation to the number of days after introduction of the queen into the colony.

Finally, the amount of eggs layed per hour (a measure of a queen's reproductive output or success) was examined. The amount of eggs layed per hour (calculated by dividing total number of eggs laid by total number of hours a queen was left in excluder) was not significantly correlated with queen age (Figure 6a; r= 0.227, n= 7, p= 0.547) or time after queen introduction to colony (measured as days after a queen was introduced into a colony with unrelated workers) (Figure 6b; r= 0.301, n=7, p= 0.513). Young queens laid an average of 13.66 eggs per hour, while older queens laid an average of

17.38 eggs per hour. The amount of eggs laid per hour did not have an obvious effect on sperm usage (r= 0.238, n= 7, p=0.608) (see Figure 7).

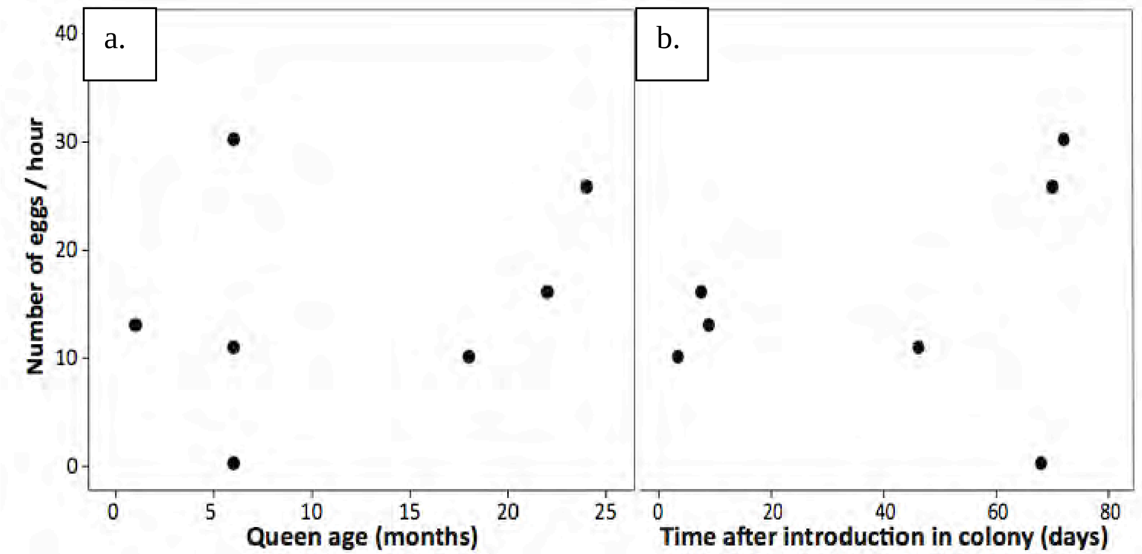

Figure 6: Number of eggs laid per hour in relation to (a) queen age in months and (b) time after queen introduction in colony in days. Each point represents the rate of egg laying of a single queen.

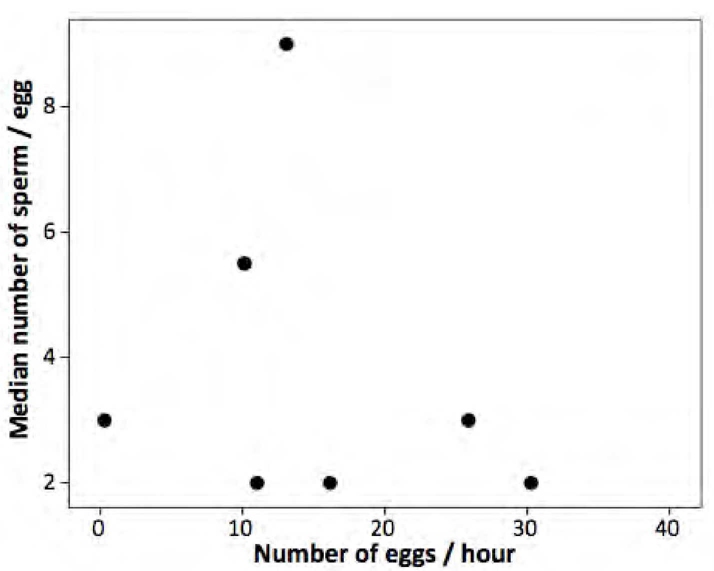

Figure 7: Median number of sperm per egg in relation to number of eggs laid per hour. Each point represents the median sperm use of a single queen.

A new technique was successfully developed to visualize sperm cells on freshly laid eggs in the honeybee *Apis mellifera*. It was shown that it is possible to collect eggs from queens, by locking queens onto a frame using a queen excluder box in their own colony. This method seems to be least stressful for the queens, as opposed to methods were queens are either completely separated from the workers or where the frame is taken out of the colony and placed in an artificial observation hive (both methods were tested, but not described here). It has been furthermore shown that it is possible to observe sperm cells on eggs that are between 0 and 2.5 hours old, and that leaving the queens alone for 2.5 hours seems to be an optimal amount of time to allow the queen to get used to being locked in the queen excluder and to harvest eggs where the sperm cells are still visible. This technique can be used to study the effects of various traits on sperm use in fundamental science and it might be important for the bee industry as well.

Discussion

Sperm Use

In general, queens are very efficient when using their sperm supply to fertilize eggs; no queen used a median of more than ten sperm, with an overall median of only three sperm per egg. This is not surprising because the queen's spermatheca holds an average of 4.7 million sperm and she lays about 1.7 million eggs in her lifetime (Baer 2005). In order to produce the highest number of fertilized eggs (female offspring), she must conserve as much sperm as possible while still guaranteeing fertilization. She must also conserve as much as possible early in her lifetime as the stored sperm reduce in viability with time stored (Locke and Peng 2008; Den Boer et al. 2009). While a correlation between queen age and sperm use was not found, this could be due to the small sample size and influence of other factors, including days after queen introduction into the colony. In the ant *Atta colombica* it was shown that sperm use increases with queen's age, and it was hypothesized that this was caused by either senescence of the sperm cells stored in the queen or of the mechanism releasing sperm from the spermatheca (queen senescence) (Den Boer et al, 2009). It would therefore be nice to repeat this experiment using more queens of different age classes and to follow the sperm use patterns of the same queens over time.

There was no significant correlation between sperm use and the time after which queens were introduced into the colonies when looking at one median for each queen, however the interaction factor between time after introduction and queen ID was significant showing that, when looking at each queen individually over time, at least some of the queens decrease their use of sperm with time after introduction. In addition, queens generally seem to use more sperm right after they were introduced into a colony with unrelated workers, compared to when they were in the colony for a longer time. This could be due to the fact that when first introduced to a colony of unrelated individuals, a queen must win their acceptance not only through the use of her pheromones, but also through the proof of her fecundity. If she was too sparing with her sperm use and accidentally laid too many unfertilized eggs in worker cells, the workers would be less inclined to accept her and would be more likely to kill her. It is possible that queen 16 slowed down too quickly or used too much sperm to fertilize the eggs at the beginning of the introduction and ran out of sperm as the colony killed her on the seventh day after introduction. Of course, queen 16 was an older queen, so she most likely had less sperm to start with compared to the younger queens. In addition, she was at an age where beekeepers would normally replace queens with a young, newly mated queen, because they are no longer consistent in laying fertilized eggs in adjacent cells ('spotty brood'). All factors combined might have made the workers decide to kill her.

The median sperm used per egg found in this study is much lower compared to estimates from earlier studies. While other studies have reported sperm use in the 20s, 50s and even 100s, this study has shown that these numbers are outliers rather than the normal trend (Tschinkel 1987; Harbo 1979; Baer 2005). This may be due to the fact that previous use was calculated by looking at number of sperm in the spermatheca after mating and then again after a certain time period, over which she was assumed to have laid a certain number of eggs. However, this study has shown that egg laying rate is extremely variable between the queens, as is sperm use per egg so that it would not make sense to look at sperm number after, for example, 10,000 eggs for one queen and 100,000 eggs for another and compare the two to calculate sperm use. By looking directly at sperm on eggs, this study was able to see the differences between the queens. It has also eliminated the need to kill queens to analyze the contents of their spermatheca, allowing one queen to be followed through different ages and introduction stages.

Rate of Egg Laying

No significant effect was found of rate of eggs laid on sperm use. While this may be due to the small data set, it may also be due to the fact that queens are very accurate at controlling their spermathecal duct and the amount of sperm released even at times when she is laying many eggs very quickly. This is an advantage as, at peak times in the year, the queen can lay up to 100,000 per hour (Baer 2005).

It was also shown that age had no effect on rate of egg laying, showing that older queens whom the colony do not reject have the capacity to lay just as well as the younger queens. Rate might actually increase with age as the older queens were found to have a higher average laying rate than the younger queens (17.38 and 13.66 respectively). In ants, sperm usage has been found to increase with age and a possible reason for this is to guarantee fertilization of eggs because of decreased sperm viability (Den Boer et al. 2009). The honeybee queens may solve the problem of decreased viability not by using more sperm per egg, but rather by laying more eggs and allowing workers to remove the unfertilized eggs. The queen must be careful, however, because the workers may kill her if too many unfertilized eggs are laid.

There was no effect of time after introduction on number of eggs laid, however there seemed to be an effect of trial number on number of eggs laid in some queens. Many queens did not lay at all or laid very few eggs the first time trialed but this number increased with trial number. This suggests that the box does create a certain level of stress for the queen, but that she can become accustomed to being in the box and feel quite comfortable, even on only the third day inside.

Suggestion for Further Study

Because of the limited time frame of this study, more data should be collected to make any conclusive statements about the effect of age on sperm use and rate of egg laying and the effects of time after introduction in a colony and rate of egg laying on sperm use. The factor of time after introduction in a colony must be considered when setting up further experiments. For example, queens of the two age groups must be settled in a colony for three or four months before trialing to eliminate influence of the introduction factor. It would also be very interesting to run a separate experiment looking at the effect of this factor in which queens would be introduced and followed throughout their settling to see if sperm use really does decrease as the queen becomes more comfortable in the colony and as the colony has more related individuals and is less likely to reject her.

Conclusion

An important method for collecting freshly laid eggs and successfully counting the number of sperm found on these eggs was developed and shown to work consistently. This study has shown that this method can be used for biological experiments and has found that individual queens differ in their average sperm use per egg fertilization and that recently introduced queen tend to use a higher number of sperm per egg. This method is very useful to evolutionary biologists not only to analyze factors affecting sperm use, but also to analyze different effects of sperm competition and other reproductive behavior, such as how queens compensate for low number and viability of stored sperm. This method could also be used to see if sperm use patterns have any hereditary components and if related queens use a similar number of sperm per egg. If this is the case, it would be extremely useful to beekeepers to be able to breed queens who use the fewest number of sperm possible without laying patchy brood (as workers remove any eggs that have been mistakenly unfertilized). This would lengthen queens' lifetimes and reduce the need for queen replacement, which costs keepers precious time and money.

References

Baer, B. (2005). “Sexual selection in *Apis* bees.” *Apidologie* 36, 187-200.

Baer, B., Armitage, S., Boomsma, J. (2006). “Sperm storage induces immunity cost in ants.” *Nature* 441, 872-875.

Den Boer, S., Baer, B., Dreier, S., Aron, S., Nash, D., Boomsma, J. (2009). “Prudent sperm use by leaf-cutter ant queens.” *Proc. R. Soc. B* 276, 3945-3953.

Harbo, J.R. (1979). “The rate of depletion of spermatozoa in the honeybee spermatheca.” *J. Apic. Res.* 18, 204-207.

Kocorek, A., Danielczok-Demska, T. (2002). “Comparative morphology of the spermatheca within the family Dinidoridae (Hemiptera: Heteroptera).” *Eur. J. Entomol.* 99, 91-98.

Locke, S., Peng, YS. (2008). “The effects of drone age, semen storage and contamination on semen quality in the honey bee (*Apis mellifera*).” *Physiological Entomology* 18, 144-148.

Paul, A.J. (1984). “Mating frequency and viability of stored sperm in the Tanner Crab *Chionoecetes bairdi* (Decapoda, Majidae).” *Journal of Crustacean Biology* 4, 375-381.

Pitnick, S., Markow, T., Spicer, G. (1999). “Evolution of multiple kinds of female sperm-storage organs in Drosophila.” *Evolution* 53, 1804-1822.

Ratnieks, F., Keller, L. (1998). “Queen control of egg fertilization in the honey bee.” *Behav. Ecol. Sociobiol.* 44, 57-61.

Snodgrass, R.E.. Anatomy of the Honey Bee. Ithaca and London: Comstock Publishing Associates, 1984.

Tschinkel, W. (1987). “Relationship between ovariole number and spermathecal sperm count in ant queens: a new allometry.” *Annals of the Entomological Society of America* 80, 208-211.

Winston, Mark. The Biology of the Honey Bee. USA: Harvard University Press, 1991.

Yu, R., Omholt, S. (1999). “Early development processes in the fertilized honeybee (*Apis mellifera*) oocyte.” *Journal of Insect Physiology* 45, 763-767.