

REVIEW ARTICLE



Standard methods for instrumental insemination of *Apis mellifera* queens

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Received 1 March 2012, accepted subject to revision 10 August 2012, accepted for publication 5 June 2013.

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Summary

Honey bee queens are highly polyandrous and mate in flight. Instrumental insemination is an essential tool that provides complete control of honey bee mating for research and breeding purposes. The technique requires specialized equipment to anesthetize and immobilize the queen and to collect and deliver semen from the drones. Semen is harvested from mature drones by hand eversion of the endophallus and collected into a syringe. The queen is placed in a chamber and anesthetized during the procedure of insertion of semen into the oviducts. Queens are introduced into colonies and their performance can equal to that of naturally mated queens, given proper technique and care.

Métodos estándar para la inseminación artificial de reinas de *Apis mellifera*

Resumen

Las reinas de las abejas melíferas presentan un elevado grado de poliandria y se aparean durante el vuelo. La inseminación artificial es una herramienta esencial que proporciona un control completo del apareamiento de las abejas con fines de investigación y de cría. La técnica requiere de un equipo especializado para anestesiarse e inmovilizar a la reina y para coleccionar y administrar el semen de los zánganos. El semen se obtiene de zánganos maduros por eversión manual del endofalo y se recoge en una jeringa. La reina se coloca en una cámara y se mantiene anestesiada durante el proceso de inserción del semen en los oviductos. Las reinas se introducen en las colonias y su rendimiento puede ser igual al de las reinas que se aparearon de forma natural, si se realiza la técnica y se da la atención adecuada.

西方蜜蜂蜂王人工授精的标准方法

摘要

蜂王高度多雄交配并在飞行时交尾。人工授精是在科研和育种中完全控制蜜蜂交尾的必要手段。该技术需要专业化设备来麻醉和固定蜂王，以及收集和释放雄峰精液。精液需通过手工外翻成熟雄峰的内阴茎采集，并收集到注射器中。蜂王放置于操作室，并在精液注入输卵管过程中处于麻醉状态。若给予适当的技术和护理，人工授精蜂王介绍到蜂群后的表现可与自然交尾蜂王等同。

Keywords: COLOSS, BEEBOOK, honey bee, *Apis mellifera*, queen, drone, insemination, valvfold, oviduct, eversion, endophallus, semen, spermatheca

Footnote: Please cite this paper as: COBEY, S W; TARPY, D R; WOYKE, J (2013) Standard methods for instrumental insemination of *Apis mellifera* queens. In V Dietemann; J D Ellis; P Neumann (Eds) *The COLOSS BEEBOOK, Volume 1: standard methods for Apis mellifera research. Journal of Apicultural Research* 52(4): <http://dx.doi.org/10.3896/IBRA.1.52.4.09>

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1. Introduction

The natural mating behaviour of honey bees presents a unique challenge to control. Queens are highly polyandrous and mate in flight with an average of 10 to 20 drones (Tarpay & Nielsen, 2002) at congregation areas consisting of 10,000 to 30,000 drones from diverse genetic sources (Koeniger 1986). Instrumental or artificial insemination (I.I.) is an essential tool that provides complete control of honey bee mating for research and breeding purposes (Laidlaw, 1977).

1.1. Applications

The technique of I.I. enables controlled mating of honey bees and provides the capability to create crosses beyond what might occur naturally. Novel crosses can be created to advance research and breeding efforts.

- A single drone can inseminate one or even several queens to isolate, enhance, and select a specific trait, which may not be expressed due to the effects of a queen naturally mating with many drones
- Semen from hundreds of drones can be pooled to inseminate a group of queens, which increases the uniformity and effective breeding population size for stock improvement and maintenance purposes

- Varying degrees of inbreeding can be created, including "selfing": the mating of a virgin queen to her own drone sons
- Provides the ability to store honey bee semen. Semen viability can be maintained at room temperature for a few weeks, for convenience in insemination scheduling and the transport of semen

1.2. Current uses

I.I. has been widely used by the scientific community for research and breeding purposes. It has been slow to be adopted by the commercial industry due to the various steps involved to realize the benefits of a breeding programme. Most successful breeding programmes have been limited to cooperative efforts between industry and research institutions with the resources to provide the varied and required expertise.

The general procedure of I.I. is to anesthetize and immobilize a queen bee, manually open her sting chamber, and inject collected drone semen into her vaginal orifice with a syringe. Beyond mastering insemination techniques, the steps required to conduct successful I.I. in honey bees include:

- applying knowledge of breeding principles
- understanding the unique challenges of working with a haploid-diploid super-organism
- practically selecting proper methods and record keeping
- access to and maintaining a large and genetically diverse breeding population (see Meixner *et al.*, 2013)
- acquiring the resources, labour, and a long-term commitment to a breeding programme, and
- learning advanced beekeeping skills, such as queen rearing and drone production (see Büchler *et al.*, 2013)

2. Equipment

The basic technique of I.I. has not changed significantly since its development in the 1950s. Proficiency requires practice, precision, and sanitary conditions. Specialized beekeeping skills and proper care of queens and drones are essential to quality control. Several options of instrumentation are currently available, which offer choice but can vary in quality and lack standardization. The basic instrument consists of a stand, a set of hooks, queen holder assembly, syringe, and syringe tips (Figs. 1, 2, and 3). The microscope stand must be compatible with the instrument and provide sufficient depth of field and instrument clearance (Fig. 1). A cold light source is also recommended to prevent heating and drying. A source of carbon dioxide with flow regulator and flexible tubing to the instrument are also required.

Equipment requirements include:

- Complete insemination instrument, including an instrument stand, manipulators, syringe, and accessories (available through specialty honey bee supply companies)
- Binocular stereozoom microscope, 10x to 20x, and cool light source
- Carbon dioxide source with flow regulator and tubing
- Saline solution (see section 2.2.)
- Sterile vials
- Pipettes and bulb or syringes
- Distilled water
- 95% ethanol
- Sodium hypochlorite
- Sterile tissues and cotton swabs
- Squeeze bottles
- Paper towels or kimwipes
- Autoclave or pressure cooker (for sterilization)
- Queen cages
- Drone holding cages and drone flight box (Fig. 4)

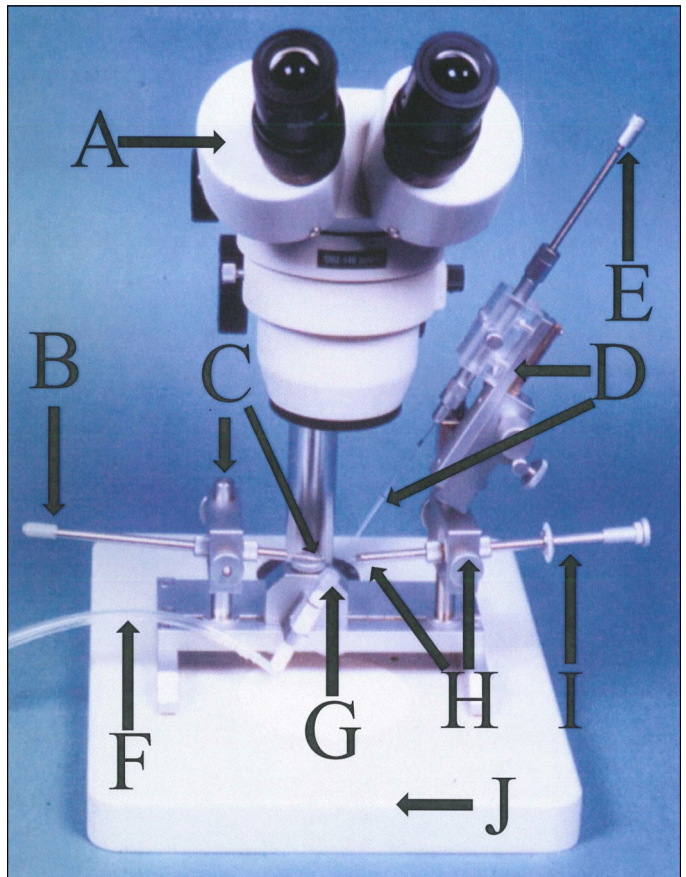


Fig. 1. Standard equipment and arrangement for performing instrumental insemination of honey bee queens. (A) a dissecting microscope, (B) handle for ventral hook, (C) ventral hook base (left C arrow) and ventral hook (right C arrow), (D) syringe base (upper D arrow) and syringe (lower D arrow), (E) syringe plunger, (F) plastic tubing leading to CO₂ source, (G) chamber in which queen is placed, (H) sting hook (left H arrow) and sting hook base (right H arrow), (I) handle for sting hook, and (J) microscope base.

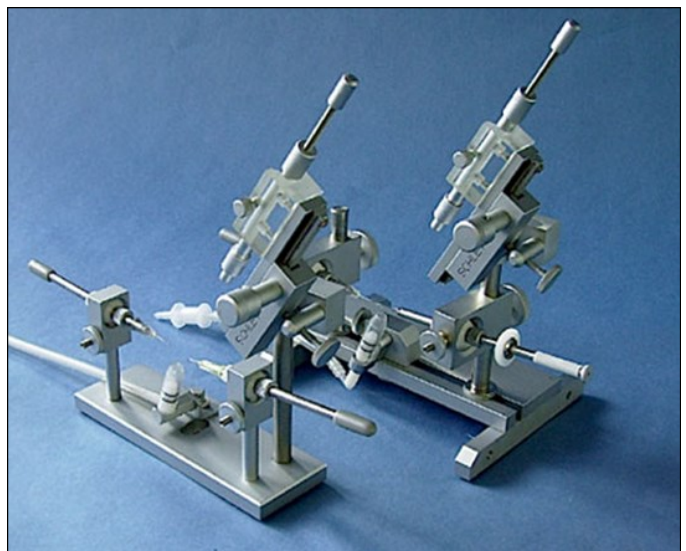


Fig. 2. Schley Instruments with micro-manipulated syringe, set of dorsal and ventral hooks, queen holder assembly with CO₂ attachment (for labelled parts, see Fig. 1).

Drone holding cages are made of queen excluder material to allow worker bees access to care for them when held in nursery colonies. Cages can vary in size, although they should be sized to fit in a frame space in nursery colonies and also fit easily in a flight box. Drones are released into the flight box for easy access during semen collection (Fig. 4).

2.1. Instrument options and choices

Instrument quality, precision, and accuracy will determine the ease and repeatability of the technique. Most instruments offer micro-manipulators that provide precision in movement and fine adjustment. Various designs of sting manipulation tools offer personal choice in techniques. Large capacity syringes provide efficiency in semen collection and a practical method of semen storage and shipment. While the protocol describing the procedure is general for all instruments, the pictures depict herein are the Schley Instrument (Fig. 2) and the Harbo large-capacity syringe (Fig. 3).

2.2. Saline diluent formulas

Two saline diluent formulas are recommended. The simple formula (section 2.2.1.) is for insemination with fresh collected semen used for insemination the same day. The second formula (section 2.2.2.) is for storage and mixing of semen (Hopkins *et al.*, 2012).

- Use double distilled water to make all solutions. Add all of the components to a volumetric flask then add distilled water to make up a final volume
- To sterilize the final product, use bacteriological filtering (pore size 0.2 μm)
 - Solutions can also be heat sterilized at $\sim 177^{\circ}\text{C}$ for 30 minutes
 - Add amino acids and antibiotic only after heating
- Adjust the pH of the final product to 8.6
 - To increase the pH, use NaOH, sodium hydroxide
 - To decrease the pH, use HCl, hydrochloric acid

2.2.1. Simple saline formula

The simple saline diluent formula (Table 1) can be used for same-day semen collection and insemination (Williams and Harbo, 1982). An alternative, very basic physiological saline solution is adequate (0.9% NaCl, 0.1% glucose and antibiotic).

2.2.2. Formula for semen storage and mixing

A more complex saline diluent formula, HHBSE Saline Formula (Tables 2 and 3), is recommended when mixing and storing semen, including storage at temperatures above freezing and in liquid nitrogen (Hopkins *et al.*, 2012).

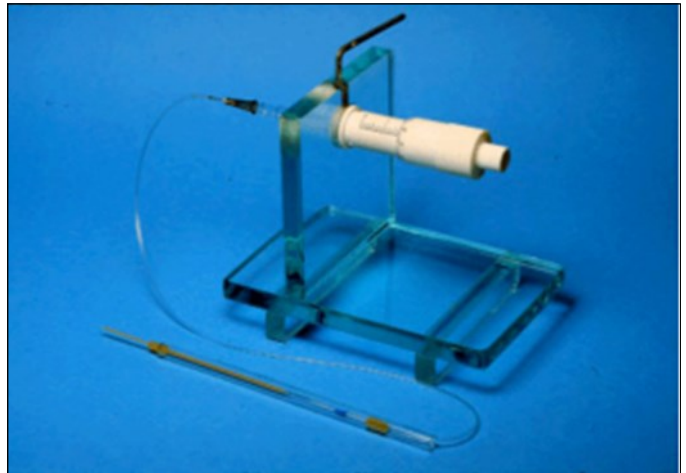


Fig. 3. Harbo large capacity syringe designed for semen collection and storage.



Fig. 4. Drone holding cage and flight box. Drones are collected in holding cages made of queen excluder material (left photograph). Cages should be sized to fit in a frame space in a nursery colony and also fit into a flight box (photograph on right: holding cage is left of white/screened flight box). Drones are released into the flight box for easy access during semen collection.

As a few of the components required (EDTA, glycine, and tylosin) are at very low concentrations and are difficult to measure on conventional scales. Consequently, it is necessary to make a 100x solution that can be added to the solution produced in Table 2 to make up the finished product.

1. Add the reagents (Table 2) (excluding EDTA, glycine, and tylosin) to the volumetric flask and fill to 100 ml (final volume) with distilled water to make the base solution.
2. The minor components, EDTA, glycine and tylosin, are made up as a 100X solution (Table 3) to be added to the solution in Table 2. Using 10 ml of the base solution (Table 2), mix in the components from Table 3. This results in 10 ml of a 100X solution of the three minor components. Then add 0.9 mL of the 100X solution back to the now 90 ml remaining of the base solution. Sterilize using bacteriological filters (pore size 0.2 μm).

Table 1. Simple saline formula based on Williams and Harbo, 1982.

Add all of the ingredients to a volumetric flask then add distilled water to make up a final volume of 100 ml.

Ingredient	g ingredient / 100 ml final volume
Dihydrostreptomycin	0.25
Glucose	0.10
L-lysine	0.01
L-arginine	0.01
L-glutamic acid	0.01
Trizma HCL	0.35
Trizma base	0.35
NaCl	1.11

Table 2. Recipe for base solution that is used in HHBSE saline solution for semen storage and mixing. Add all of the ingredients (excluding EDTA, glycine, and tylosin) to a volumetric flask, then add distilled water to make up a final volume of 100 ml. Use 10 ml of this solution to make the solution in Table 3. Add 0.9 ml of the resulting solution from Table 3 back to the 90 ml of the remaining base solution (presented here) to make the final product. *Tylosin, EDTA, and glycine compose only a small amount of the finished solution. Consequently, it is necessary to make a 100x solution of these ingredients to add back to the rest of the ingredients listed here. See Table 3 and section 2.2.2. for details.

Ingredients	g ingredient / 100 ml final volume
Penicillin	0.05
Streptomycin	0.044
Kanomyosin	0.06
Tylosin*	0.0032
EDTA (ultra pure)*	0.0002923
TES (acid)	0.6879
Tris (base)	0.3635
Sodium Phosphate Dibasic	0.0142
Sodium citrate	0.02942
Arginine	0.01
Glycine*	0.00075
Proline	0.05
Catalase	0.002
BSA (lipid rich)	0.002
KCl	0.61131
NaCl	0.4847
NaHCO ₃	0.042

Table 3. Recipe for 100x solution of ingredients that compose a small amount of the HHBSE saline solution (in Table 2). Pipette 10 ml of the solution from Table 2 to mix with the components listed below. Add 0.9 ml of this solution to 90 ml of the base solution (in Table 2) to create the final HHBSE saline solution.

Ingredients	g ingredient / 10 ml final volume
Tylosin	0.032
EDTA (ultra pure)	0.003
Glycine	0.0075

3. Insemination Techniques

3.1. Eversion of the endophallus

Semen is collected directly from mature drones, 14 days post-emergence or older. For identification purposes, drones can be collected immediately after emergence (i.e. capturing “fuzzy” drones that are newly enclosed) and stored in cages placed in a bank colony (another honey bee colony that will tend the drones; see Büchler *et al.*, 2013 for a discussion of “bank” colonies). Mature drones can be captured the day prior to or the day of insemination by capturing drones returning from failed mating flights or collecting them from the outside combs within the colony. To expose semen, the endophallus is readily everted by hand in two-steps: the partial eversion, and the full eversion.

Maintain sanitary conditions, as drones often defecate during the procedure. Hold the drone to avoid the endophallus touching the drone body or your fingers and keep a towel soaked in alcohol to clean up. The eversion of the endophallus is performed within a few seconds. Evaluation of drone maturity and semen quality must be determined instantly; any drone that does not evert properly or does not present sufficient (~1 µl) semen on the bulb (see section 3.2) should be discarded. Semen collection is tedious, therefore proper techniques and practice will greatly increase efficiency. Plan to have a plentiful supply of mature drones, more than is needed, as not all will yield semen. Keep drones warm and well fed until they are used. A light above the flight box provides warmth and bee candy or a piece of honey comb will extend their activity.

Procedure for everting drones:

1. Assemble and prepare the syringe
 - a. All parts should be sterilized, either by heat or an alcohol wash, and rinsed with distilled water.
2. To obtain partial eversion, grasp the head and thorax of the drone between the thumb and forefinger, ventro-dorsally, with the abdomen facing upward. It is helpful, if the individual is right-handed, to hold the drone’s head with the right hand and squeeze the abdomen with the left so that the drone remains held in the left in position for sperm collection.
3. Roll or crush the thorax between your fingers.
 - a. If mature, the abdomen will contract and a pair of yellow-orange cornua emerge (Fig. 5).
 - b. If the abdomen remains soft or the cornua lacks colour, the drone is immature and will not yield semen (Fig. 6).
4. To obtain full eversion, grasp the base of the abdomen near the thorax with the thumb and forefinger and apply pressure along the sides of the abdomen, starting at the anterior base and working toward the posterior tip.
 - a. Squeeze and roll your fingers together in one steady forward motion, forcing the eversion to complete.



Fig. 5. Partial eversion of the endophallus of a mature drone (left) and full eversion (right). At the stage of the partial eversion, the abdomen will contract and a pair of yellow-orange cornua appear.



Fig. 6. Partial eversion of the endophallus of an immature drone. The abdomen is soft and the cornua lacks colour.



Fig. 7. Semen contaminated. Avoid contamination of semen during the eversion process. Position the drone to stop the endophallus from falling back onto your fingers or the drone's abdomen.

- b. Hold the drone with his abdomen pointing downward to keep the endophallus from falling back onto your fingers and contaminating the semen as in Fig. 7. This positioning also provides ready placement under the microscope.
- c. The exposed semen is a creamy, marbled tan colour, with an underlying layer of white mucus (Fig. 8).

3.2. Semen collection

Semen is collected directly from the endophallus of many drones into a syringe and stored in glass capillary tubes. The amount and consistency of semen obtained from each drone varies and depends on skill and experience. Generally, each drone will yield approximately 1 μl of semen. The standard volume of semen to inseminate one queen is ~ 8 to 12 μl . Maintain sanitary conditions, as drones often defecate during eversion. It is recommended to have a paper towel readily available to wipe drone faeces. Although less common, queens may also defecate. Discard the queen if this happens during the procedure.

Procedure for collecting semen:

1. After assembly of the syringe, collect an air space (~ 5 μl) to separate the saline and semen column in the syringe. Then collect a small drop of saline into the glass tip (~ 2 μl). This will be the last fluid to be injected into the last queen inseminated, which will help wash any remaining semen adhering to the capillary walls out of the tip and into the queen.
2. Collect another small air space and collect a small drop (0.5 μl) of saline in the syringe tip (Fig. 9). Use the drop of saline to make contact with the semen on the endophallus of the first drone.
3. Skim the semen off the mucus layer and draw it into the syringe (Fig. 10).

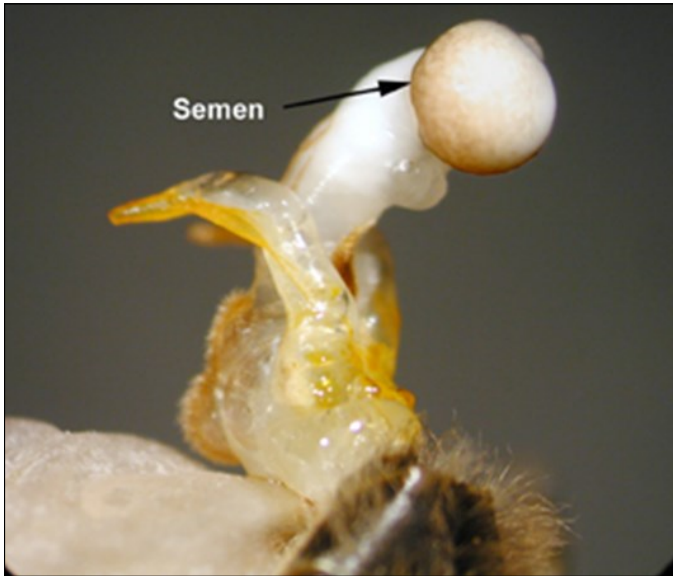


Fig. 8. Full eversion of the endophallus with semen exposed. The exposed semen is a creamy, marbled tan colour, with an underlying layer of white mucus.

- a. Avoid collecting the viscous mucus layer at all costs. If resistance is felt, back off or expel any mucus in the tip. Excess mucus in the tip can leave it clogged (Fig. 11).
- 4. Repeat Step 3 until the total desired amount of semen is collected.
 - a. Expel a small drop of semen from the syringe tip on to the semen load of the next drone and draw semen into the syringe (Fig. 12)
 - b. Avoid collection of air bubbles and additional saline in the semen column. The column of semen should be uniform in colour and density.
 - c. Between semen loads, keep the tip moist with saline to prevent drying, taking care not to excessively dilute the semen. Drone semen quickly dries and the sperm die when exposed to air.



Fig. 9. Collecting semen into the syringe. Collect an air space to separate the saline and semen column in the syringe. After the air space, collect a small drop of saline in the syringe tip to make contact with the semen on the endophallus of the first drone.



Fig. 11. Accidental collection of mucus into the syringe. Avoid collecting the viscous mucus layer as pictured here. If resistance is felt, back off or expel any mucus in the tip to avoid clogging the tip.



Fig. 10. Collecting semen. Skim the semen, a marbled tan colour, off the underlying layer of viscous white mucus and draw this into the syringe.



Fig. 12. Collecting semen from subsequent drones. To repeat the process of semen collection from the next drone, expel a small drop of semen from the syringe tip onto the semen load of the next drone and draw this into the syringe.

3.3. Insemination of the queen

Inseminate queens between 5 and 12 days post-emergence. Carbon dioxide is used to anesthetize the queen during the procedure and also stimulates oviposition. Queens can be emerged in a queenless bank or, preferably, in their own colonies (typically small hives each with several hundred adult workers with a single virgin queen, called mating nuclei). If mating nuclei are used, cage the queen cells (so queens emerge into a cage) or be sure that the hive entrances are covered with queen excluder material to prevent unwanted natural mating flights. Queenless banks, mating nuclei, and caging queens cells are all discussed in detail in B uchler *et al.*, 2013.

Procedure for inseminating queens:

1. Two CO₂ treatments are usually required. Give the first CO₂ treatment, a 1 to 4 minute exposure, one or two days before the insemination procedure. The dose can be applied by individually caging queens and placing them in a jar or plastic bag filled with CO₂. The second treatment is administered during the procedure.
2. Align the syringe and queen holder on the instrument stand at a 30° to 45° angle (dependent upon the instrument used) to facilitate bypassing the valvelfold (Figs 13 and 18).
3. Place the queen in the holding tube abdomen first, ventral side up, with her abdomen protruding several segments (Fig. 13), and administer a slow continuous flow of CO₂.
4. Separate the abdominal plates to expose the vaginal orifice using a pair of hooks or forceps (Fig. 14).
5. Lift the sting structure dorsally, to expose the vaginal cavity (Figs 15-17).
 - a. During this manipulation, position the ventral hook only to stabilize the queen.
6. Position the syringe tip dorsally above the "V", defining the vaginal orifice. Insert the tip into the vaginal orifice 0.5 to 1.0 mm, slightly forward of the apex of the "V" (Fig. 18).
7. Insert the tip further, another 0.5 to 1.0 mm, while using the tip to lift the valvelfold ventrally (Fig. 18). Use a slight "zigzag" movement to bypass the valvelfold.
 - a. The valvelfold, a stretchy flap of tissue covering the median oviduct, must be bypassed or semen will back-flow out of the vaginal orifice.
 - b. Correctly inserted, the tip slips easily past the valvelfold without resistance.
 - c. As outlined in section 3.2., it is useful to leave a tiny air bubble between the saline and semen and release some of the saline for lubrication before inserting the tip in the queen.

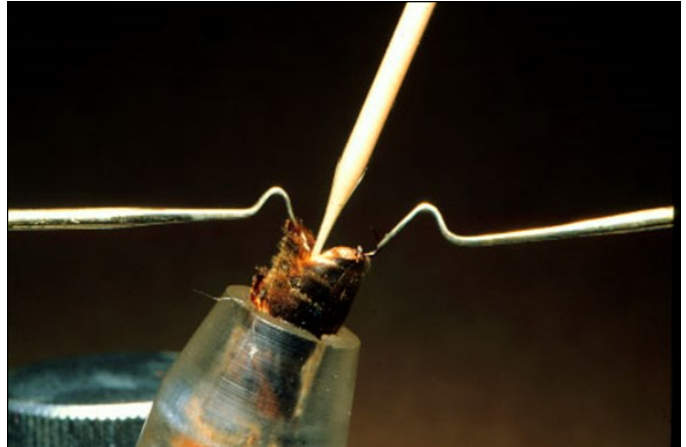


Fig. 13. Virgin queen positioned in the holding tube. The syringe and queen holder are aligned at a 45° angle on the device to facilitate bypassing the valvelfold.



Fig. 14. Separating the abdominal plates of the queen to expose the sting structure using the perforated sting hook. The ventral hook is on the left while the perforated sting hook (seen with small hole to accommodate the sting) is on the right.

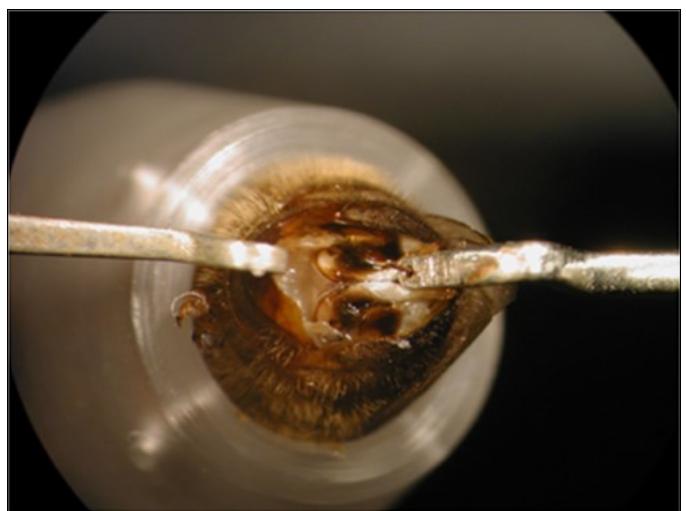


Fig. 15. Threading the sting through the perforated sting hook (on right). The ventral hook is on the left and is used only to stabilize the queen.



Fig. 16. Lifting the sting structure to expose the vaginal cavity, using a perforated sting hook (on right).



Fig. 17. Lifting the sting structure, using Schley pressure grip forceps (on right).

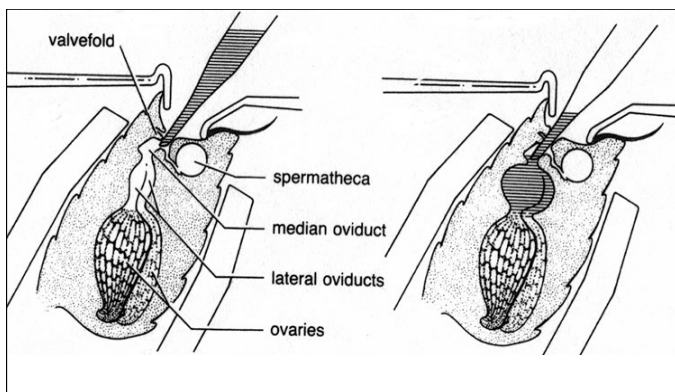


Fig. 18. Bypassing the valvefold. To bypass the valvefold, position the syringe tip dorsally above the "V", defining the vaginal orifice. Insert the tip about 0.5 to 1.0 mm, slightly forward of the apex of the "V". Insert the tip another 0.5 to 1.0 mm lifting the valvefold ventrally, using a slight "zigzag" movement to manoeuvre around the valvefold.

8. Deliver a measured amount of semen directly into the median oviduct (Fig. 19).
 - a. The standard dosage is 8 to 12 μl per queen. When giving a 12 μl semen dose, release the queen directly into her mating nucleus colony to promote sperm migration or give two 6 μl semen doses 48 hours apart.
 - b. With practice, the insertion of semen is preformed quickly and precisely, requiring only seconds per queen.
9. After insemination, remove the syringe tip, collect a small air space and small drop of saline, ($\sim 0.5 \mu\text{l}$) to precede the next insemination.
 - a. Keep a drop of saline in the tip to prevent any residual semen from drying and to initiate subsequent semen collection.
 - b. If inseminating queens with different drones where precise genetic crossings are paramount, rinse the insemination tip with distilled water then saline to completely cleanse the syringe of semen from the previous drone.
10. Release the queen from the holder, place her in a cage, and return her to her nucleus colony.

3.4. Field dissection of the spermatheca

When learning the procedure of insemination, it is helpful to check the spermatheca to determine the degree of insemination success. Sperm migration requires about 40 hours post insemination. After insemination, a subset of queens can be held in a nursery colony until tested.

Procedure for field dissection of spermatheca:

1. Sacrifice the queen, by crushing her head and thorax.
2. Grasp the queen's terminal abdominal segments, dorsally and ventrally.
3. Pull and separate the terminal segments from the rest of the queen's body, with your fingernails or forceps (Fig. 20).
 - a. The spermatheca is a white, spherical structure about 1 mm in diameter, and appears rough in texture due to the trachea net covering (Fig. 21).
4. Tease the spermatheca out of the body cavity with your thumbnail or forceps.
5. To remove the tracheal net, gently roll the spermatheca between your fingers. The net will collapse in a small white mass.
 - a. The colour shade and density of the spermatheca indicates the relative insemination success of the queen.
 - b. The spermatheca of a virgin queen is clear (Fig. 22).
 - c. A cloudy or milky appearance of the spermatheca indicates an inadequate insemination or a failing queen (Fig. 23).
 - d. For a fully inseminated queen, the spermatheca is a creamy tan colour with a pattern of marbled swirls (Fig. 21).



Fig. 19. Insertion of semen into the median oviduct. Positioned correctly, the tip slips easily past the valvefold without resistance.

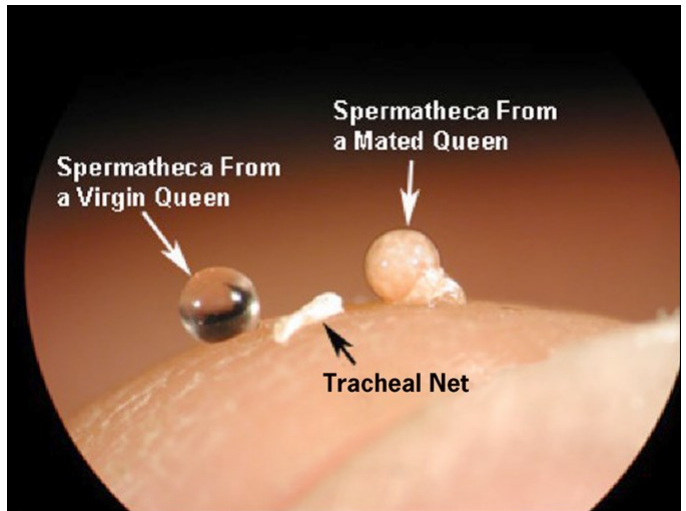


Fig. 22. Comparison of spermathecae of a virgin queen (clear) and mated queen (tan with a pattern of marbled swirls).

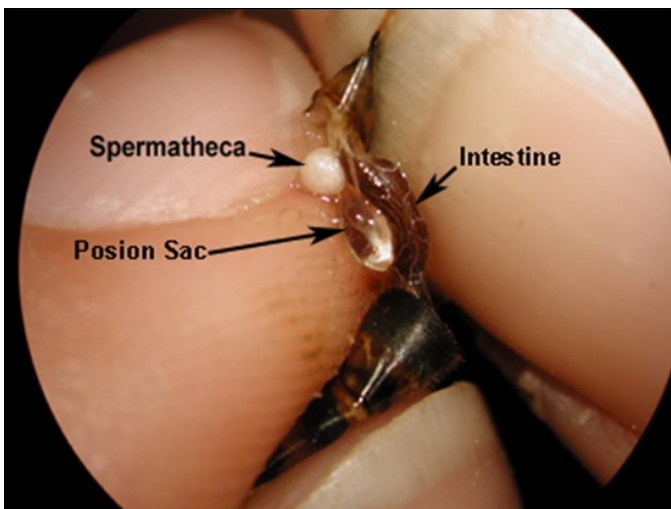


Fig. 20. Exposing the spermatheca. To expose the spermatheca, grasp the queen's terminal abdominal segments, dorsally and ventrally and pull to separate these segments from the rest of the queen's body, with your fingernails.



Fig. 23. The spermatheca of a failing or poorly mated queen is a milky colour.

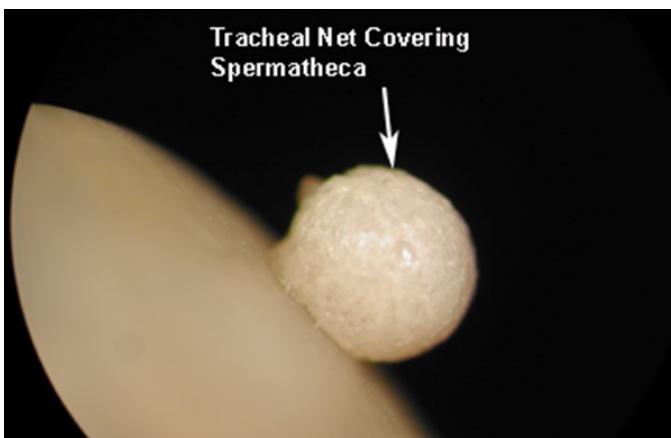


Fig. 21. The spermatheca is a white, spherical structure about 1 mm in diameter and appears rough in texture due to the trachea net covering.

4. Maintenance of queens and drones and factors affecting queen performance

Given proper care, instrumentally inseminated queens are capable of heading productive colonies and enduring the rigors of performance testing in the field (Cobey, 2007). Many factors that influence queen performance can be optimized through proper beekeeping management practices. The quality of the insemination, in terms of technique and sanitation, are critical. The treatment of queens before and after the insemination will influence the amount of semen stored and queen performance. Natural conditions should be maintained as much as possible.

4.1. Maintenance of drones for instrumental insemination

Producing a large number of mature drones from select sources can be more challenging than queen rearing, especially if seasonal conditions are not optimal. Drones have a high rate of attrition and drift heavily among colonies. Free flying drones have better survival and have a prior opportunity to void faeces.

1. Use strong, healthy, well-fed colonies for drone production.
 - a. Colonies headed by older queens tend to rear and maintain more drones.
 - b. Minimize colony stressors, especially exposure to pathogens, parasites and chemical residues (see the *BEEBOOK* papers on pests and diseases: Dietemann *et al.*, 2013; Forsgren *et al.*, 2013; Fries *et al.*, 2013; de Graaf *et al.*, 2013; Jensen *et al.*, 2013; de Miranda *et al.*, 2013; Sammataro *et al.*, 2013).
2. Drones require 12 -14 days post-emergence to sexually mature (Woyke and Jasinski, 1978).
3. Maintain the identity of drone sources.
 - a. For free-flying drones, newly emerged drones can be emerged from field-collected combs placed in an incubator set at broodnest conditions (34°C, ~50% RH), marked, and returned to colonies to reach sexual maturity. Mark several times the number needed to ensure an adequate supply.
 - b. To confine drones, emerge drone brood above a queen excluder in a healthy, strong colony. Screening the top box (i.e. replacing the colony lid with screen mesh) may be used to collect the drones as mature drones are attracted to light.
4. Collect drones in holding cages made of queen excluder material (Fig. 4).
 - a. Drone flight times vary with the season and weather conditions. Watch the colonies in the local area to determine drone flight time.
 - b. Caged drones can be banked in queenless nursery colonies or queen cell builders (see Büchler *et al.*, 2013 for more information on queen cell builders).
 - i. Minimize banking time as drones are perishable, overnight to a few days.
 - ii. Banked drones accumulate faeces that need to be voided.
5. Release mature drones into a flight box for semen collection (Fig. 4).
 - a. Bring in only the number of drones that can be collected in about 30-40 minutes (100 to 150), as inactive drones are difficult to evert.

- b. Providing heat (a light above the flight box) and food (bee candy) will extend the active period of drones.

4.2. Maintenance of queens before and after instrumental insemination

The pre- and post-insemination treatment of queens will influence their performance. Maintain queens with a high proportion of nurse bees in well-fed nursery and/or nucleus colonies (see Büchler *et al.*, 2013). Direct release of queens into colonies after insemination enhances sperm migration (Woyke, 1983).

1. Optimize queen rearing conditions.
 - a. Rearing conditions influence queen size, the number of ovarioles, and spermatheca capacity (see Büchler *et al.*, 2013).
2. Place mature queen cells in individual cages before emergence.
 - a. Emerge virgins in a nursery colony or incubator.
 - b. After emergence, remove their wax cells to prevent queens crawling into them and dying.
3. Cage each newly emerged virgin queen individually in a nucleus colony for several days (5-14) before insemination.
 - a. Queens banked in a nursery colony can be subject to injury by workers resulting in damaged legs and tarsi (Woyke, 1988).
 - b. If banking is necessary, use a well-fed queenless colony maintained with open brood (eggs and larvae) and a high proportion of young nurse bees.
4. After insemination, release each queen directly into her established nucleus colony. Use a spray of sugar water (preferably scented with anise oil some other fragrant) or a small candy plug in a queen cage to facilitate introduction.
 - a. Note the behaviour of the queen upon release. If aggressive worker behaviour is observed, cage the queen and provide a slow release (i.e. release her 3-4 days later and observe worker behaviour; repeat if necessary).
 - b. If queens have been banked, use a screened push in cage (Fig. 24) or another similar slow-release method (see Büchler *et al.*, 2013).
 - c. To prevent unwanted natural mating flights of I.I. queens (Woyke and Janinski, 1992), place queen excluder material on the colony entrance until eggs are observed.
5. Allow queens to build their own populations naturally.
 - a. I.I. queens are slower than naturally mated queens to develop their full pheromone blends and tend to supersede when placed in large colonies initially.



Fig. 24. A queen “push-in” cage. These cages can be used to introduce newly-inseminated queens into colonies. To use, place the queen on a section of capped/emerging brood. Push the cage around the queen and brood, firmly into the wax comb. Leave queen in cage with emerging brood for 3-4 days. Following this time, the cage can be removed and the queen released into the colony. If eggs are not observed at release, place a queen excluder over the colony entrance until the queen begins laying.

4.3. Factors influencing results of instrumental insemination

1. The optimal age for insemination of queens is 5 to 14 days post emergence.
 - a. Queens inseminated older than 2 weeks tend to store less sperm in their spermathecae. Queens inseminated less than 4 days old have high mortality (Woyke and Jasinski, 1976).
2. The standard semen dosage given to each queen is 8 to 12 μ l.
 - a. An insufficient semen dose can result in premature queen supersedure or premature queen failure.
3. Post-insemination care of queens influences sperm storage (Woyke 1979).
 - a. Active movement of queen, appropriate brood nest temperatures, and attendance by worker bees promote sperm migration into the queen’s spermatheca.
 - b. Queens confined in cages after insemination tend to store less sperm and retain semen in their oviducts.

5. Specialized techniques

5.1. Homogenizing honey bee semen

To homogenize or mix honey bee semen from numerous drones requires dilution, mechanical movement and reconstitution of semen. Current techniques using centrifugation result in a high percentage of damaged spermatozoa, although 50% viability of spermatozoa is sufficient to produce normal brood patterns (Collins, 2000). Semen is very dense, tends to clump, and the long, fragile tails of spermatozoa are subject to damage during processing and some components of the seminal fluid are removed.

Migration of sperm from the oviducts into the spermatheca is a complex process involving contraction of muscles mediated by the specialized composition of fluids in the semen and the oviduct as well as active sperm movement (Koeniger, 1986). Queens are very active after natural mating which also promotes sperm migration; therefore, use a direct queen introduction release method (Büchler *et al.*, 2013).

Procedure by centrifugation:

1. Collect semen into glass capillary tubes (section 3.2).
 - a. Collect 10 μ l for each queen to be inseminated.
2. Add diluent (the recipe can be found in Table 4) to Eppendorf tube.
 - a. Ratio should be 10 parts diluent to 1 part semen by volume.
 - b. Up to 700 μ l of semen can be added per Eppendorf tube.
3. Expel semen into an Eppendorf tube.
4. Mix semen by inversion, gently shaking, until suspension is uniform.
 - a. Strong mixing with a Pasteur pipette may damage spermatozoa.
5. Centrifuge at a 45° angle until a semen pellet is formed.
 - a. Use speeds of 82 or 250 g at 20–30 or 10–20 min, respectively.
 - b. Higher speeds can damage tails (see Collins, 2003).
6. Carefully remove supernatant.
7. Draw semen into syringe and use immediately.
8. Inseminate queens with the required dosage.

Table 4. Recipe for making desired volumes of diluent for counting sperm. Mix the ingredients together and add distilled water to achieve the final volume.

Reagents	Desired Volume			
	2 ml	5 ml	10 ml	20 ml
1M HEPES	20 μ l	50 μ l	100 μ l	200 μ l
NaCl	0.017 g	0.0425 g	0.085 g	0.17 g
BSA	0.20 g	0.50 g	1.0 g	2.0 g
add water to bring total volume to:	2.0 ml	5.0 ml	10.0 ml	20.0 ml

5.2. Short term semen storage at above freezing temperature

Honey bee semen can be held at room temperature for several weeks without significant loss of sperm viability. The Harbo syringe, with detachable capillary tubes, is designed for semen storage (Fig. 25). For step by step pictorial instructions, see: honeybeeinsemination.com/uploads/HarboSyringeAssembly.pdf honeybeeinsemination.com/uploads/HarboSemen_Storage.pdf

5.2.1. Sealing semen-filled capillary tubes

1. Collect semen into glass capillary tubes of the syringe.
2. Remove glass tip and detach the filled capillary tube.
3. Force petrolatum into one end of the tube (~7 mm).
 - a. Insert the capillary tube into the petroleum jelly perpendicularly several times until a sufficient plug is formed.
 - b. The petroleum can be touching the semen or a small airspace can be collected between the semen and petrolatum seal.
4. Reconnect the sealed end of the tube to the syringe and push the column of semen forward to allow space to seal the other end.
5. Detach and place a petrolatum seal in the other end of the filled capillary tube.
6. An alternative method, a glass bead connected with a small piece of silicone tubing, can also be used to seal the capillary tube. The glass bead is made by heating a small piece of capillary tube to seal both ends.
7. Store in the dark at a temperature of 20°C. Avoid sunlight and temperature fluctuations.

5.2.2. To remove the petroleum seal:

1. Assemble the syringe and load the capillary tube without the glass tip.
2. Push out the petrolatum seal with the action of the syringe.
3. Attach the glass tip and pick up a small drop of saline to precede the first insemination.
 - a. Leave the seal at the terminal of the capillary tube.
 - b. After inseminations are complete, collect saline to move the seal out of the tip and up into the capillary. Discard the capillary tube.

5.3. Cryopreservation of semen

The maintenance of honey bee stocks currently requires costly and labour intensive annual propagation. Current threats to the biodiversity of honey bees, and the need to select lines tolerant to pests and diseases, creates a need to develop techniques for the cryopreservation of honey bee germplasm. Repositories

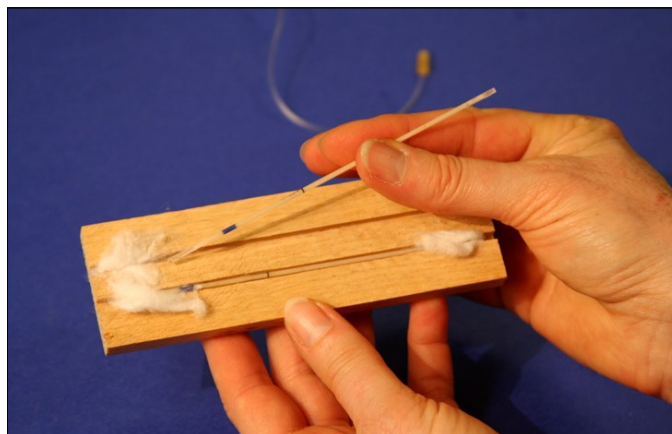


Fig. 25. Sealed capillary tubes of semen ready for transport.

would provide a resource for breeding purposes and the preservation and recovery of selected stocks and endangered populations.

High viability and motility of honey bee semen cryopreserved in liquid nitrogen and thawed has been demonstrated, although fertility is greatly reduced in the spermatheca of queens. Current techniques demonstrate that fertility is adequate to produce sequential generations of queens inseminated with frozen-thawed semen for breeding purposes, although they are not sufficient to head productive colonies (Hopkins *et al.*, 2012). Further research is being conducted to perfect these techniques.

Current recommendations for cryopreservation:

- Cryoprotectants: dimethyl sulfoxide (DMSO) and ethylene glycol (see Wegener & Bienfeld, 2010 and Hopkins and Herr, 2010)
- Programmable freezing rate: 3°C / min, from 4°C to -40°C, then place samples in liquid nitrogen (see Hopkins *et al.*, 2012).
- Thawing rate, 40°C for 10 seconds (see Hopkins *et al.*, 2012)

5.4. Techniques for counting sperm

5.4.1. Queen spermathecae

1. Place the test queen in a freezer (-20°C is sufficient) 4-6 mins or until immobilized.
2. Remove the queen from freezer and weigh to the nearest 0.1 mg on digital scale. Measure the queen's thorax and head using micro-calipers to the nearest 0.1 mm and record. *NOTE: Non-destructive morphometric measures such as these may be helpful and potentially important correlates of other measures of queen reproductive potential (see Delaney *et al.*, 2011).
3. Dissect out the spermatheca:
 - a. Euthanize the queen by removing her head and pin her body to a dissection tray.

- b. Cut her abdomen along both sides.
- c. Grasp stinger with forceps and gently pull out until the ovaries are exposed.
- d. Gently push hindgut aside to reveal the spermatheca (off-white, semi-hard sphere; see Fig. 21).
4. Carefully pull the spermatheca out and place on a watchglass. Remove the tracheal netting covering it if still attached.
5. Set dissection microscope to maximum magnification, and use graduations on ocular to measure spermatheca diameter. Measure two diameters and record the average. This is an optional but potentially useful measure for spermatheca volume, which can be used to calculate the theoretical maximum storage capacity and therefore the percentage filled (see Tarpy *et al.*, 2011).
6. Place spermatheca in 0.5 ml sperm diluent (e.g. Table 4) in a small glass beaker, then use forceps to burst it to release sperm.
7. Immediately place remainder of queen in a labelled 1.5 ml microcentrifuge tube and place in -80°C freezer for any further analyses (e.g. PCR, GC-MS, etc.).

5.4.2. Drone seminal vesicles

1. Cut off the drone abdomen and immediately freeze head/thorax at -80°C for any further analyses.
2. Pin abdomen down in petri dish and dissect out the seminal vesicle:
 - a. Cover with 0.9% saline solution, then make ventral incisions.
 - b. Pull the two cut sides apart and grab the bursa, then gently pull out until mucous glands and seminal vesicles come with it.
 - c. Cut seminal vesicles away from mucous glands.
3. Place seminal vesicle into a 50 ml beaker containing 1 ml sperm diluent (see Table 4 for recipe).

5.4.3. Sperm count protocol (unstained sperm)

This protocol is covered in some detail in Human *et al.*, 2013. Despite that, we include an expanded version of the technique here as we list details specific to counting sperm.

1. Dissect out the spermatheca of a mated queen or obtain the sperm sample from a desired drone (queen: section 5.4.1., drone: section 5.4.2.). *NOTE: Always use glassware when dealing with sperm, because they can stick to plastic.
2. Add 10.0 ml of diluent or water to a glass petri dish. *NOTE: water causes live sperm to contract and contort their shape, so only use water with frozen or dead sperm.
3. Break open the spermatheca using forceps, taking care to remove all sperm, and remove the remaining tissue.
4. Mix well with a clean glass pipette about 40 times until all

sperm are dispersed. Use caution to prevent air bubbles or excess splattering.

5. Immediately add a drop of the diluent/sperm mixture to both sides of a haemocytometer on which a cover slip has been placed. Capillary action will fill each chamber (the area between the cover slip and slide) with solution. View under 250× magnification. Start counts on the gridded section after the sperm have settled (~ 20 seconds).
6. The sperm appear headless, translucent, and filamentous. They are usually about 0.25 mm long, but are often coiled or looped. It is best to bring the focus slightly upwards from the grid and to keep the light somewhat dim in order to best see them. Rapidly change the fine-tuning focus on the microscope to observe those that are not laying on the bottom of the haemocytometer.
7. Count the number of sperm in five of the large 1.00 mm² squares in the grid, preferably the large squares in the four corners and the one in the centre (5 bold, black squares seen in Fig. 26). The centre square will contain a smaller grid (used to count red blood cells at a greater magnification), while the remaining squares will be divided into 16 squares (4 × 4 grids). *NOTE: There are different types of haemocytometers so it is important to follow manufacturer's directions when using one as calculations and haemocytometer volume may differ between types. Our calculations are done using a Bright-Line haemocytometer (Hausser Scientific).
8. Since sperm often overlap the boundaries of the squares, only count those sperm which are entirely within a square or are *only* on the top and left boundaries (or bottom and right, if you prefer). This procedure will prevent double counting of sperm and give a more accurate count.

Calculate the total number of sperm in 5 large squares in *both* chambers of the haemocytometer. Divide the total number of sperm in the 10 large squares (5 large squares per 2 chambers) by 100 to estimate the number of millions of sperm. (The volume of each large square is 1.0 mm × 1.0 mm × 0.1 mm = 100 nl, making the total volume counted for ten squares = 1000 nl, Table 5. Thus the number of sperm, for an initial dilution of 10.0 ml, is to the order of 10⁴ which is equivalent to 10⁻² million).

Alternative method for counting sperm:

In this method, the sperm are induced to coil in the solution, which facilitate their counting (Woyke 1979).

1. Add a drop of saline solution into a small porcelain evaporator, preferably one having blue bottom.
2. Break open the spermatheca, remove all sperm, and remove the remaining tissue.
3. Stir with the dissecting needle and add more solution up to a total of 1 ml.

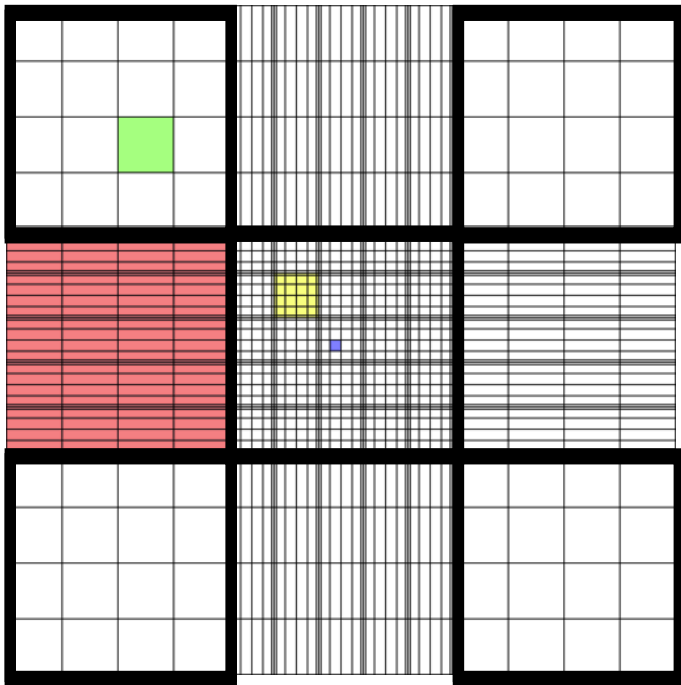


Fig. 26. Haemocytometer grid. Red square (and each of the 5 large squares with bold, black lines added) = 1 mm² (100.00 nl); green square = 0.0625 mm² (6.250 nl); yellow square = 0.040 mm² (4.00 nl); blue square = 0.0025 mm² (0.25 nl). All squares are at a depth of 0.1 mm. For area and volume calculations per certain grid dimensions on the haemocytometer, see Table 5. Information is for Bright-Line Haemocytometer (Hausser Scientific). Figure from Wikipedia.

Table 5. Area and volume calculations for Haemocytometer grids seen in Fig. 26. The volume is calculated as L × W × D with the L and W provided in the “Dimensions” column and the D set as 0.1 mm with the standard Haemocytometer (such as a Bright-Line Haemocytometer, Hausser Scientific).

Dimensions	Area	Volume
1 × 1 mm (1 red and the 5 black bolded squares in Fig. 26)	1 mm ²	100 nl
0.25 × 0.25 mm (green square in Fig. 26)	0.0625 mm ²	6.25 nl
0.25 × 0.20 mm (square not highlighted in Fig. 26)	0.05 mm ²	5 nl
0.20 × 0.20 mm (yellow square in Fig. 26)	0.04 mm ²	4 nl
0.05 × 0.05 mm (blue square in Fig. 26)	0.0025 mm ²	0.25 nl

4. Add 9 ml of tap water, which will result in a total of 10 ml of solution, and mix well. The tap water causes the sperm to coil.
5. Mix well with a clean glass pipette until all sperm are dispersed. Use caution to prevent air bubbles or excess splattering.
6. The Fuchs-Rosenthal count chamber is used to count the sperm (Fig. 27). There are two counting grids and the depth of the counting chamber is 0.2 mm.

7. Add a drop of the diluent with sperm on both grids and cover with a cover slip.
8. Wait for the sperm to settle.
9. Use 250× magnification for counting the sperm.
10. Count the number of sperm in 5 large 1 mm² squares on both grids (totalling 10 squares). The sperm will be easy to count because they will be coiled. Some sperm will be located over the square boundary. To avoid double counting, count only those that are over the top and left boundaries.
11. Calculate the total number of sperm as follows: the total volume of the solution over the 10 large squares (5 squares in both grids) in which the sperm are counted is: 1 × 1 × 0.2 × 10 = 2 mm³. However, the total volume of the solution in which the sperm were dispersed is 10 cm³ = 10,000 mm³. Thus, the volume of the dispersion solution is 10,000:2 = 5,000 times higher. To get the total number of spermatozoa, multiply the number of spermatozoa counted over the 10 large squares by 5,000.

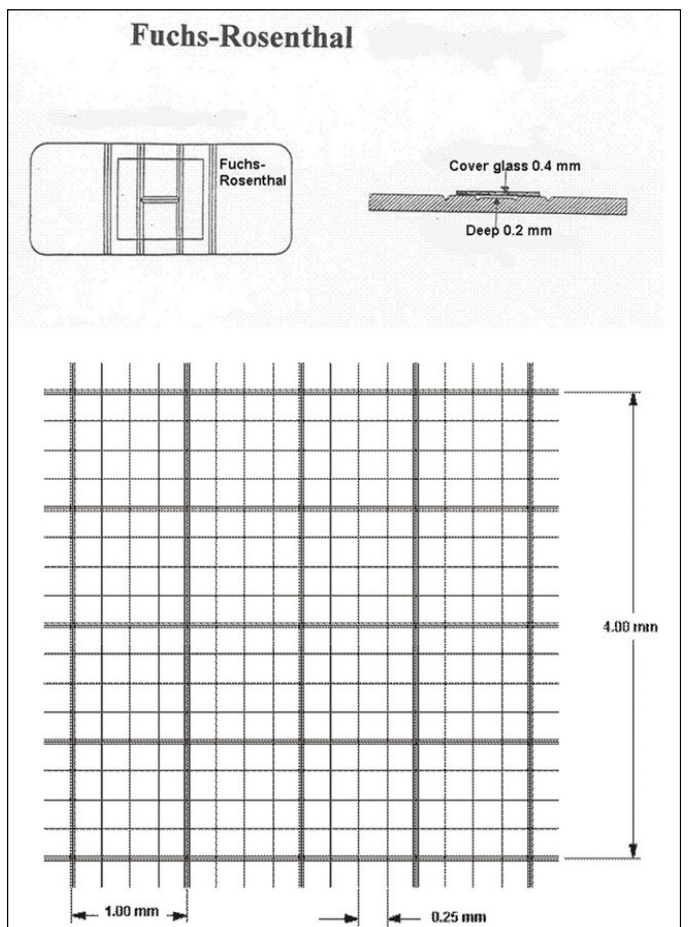


Fig. 27. Diagram of a Fuchs-Rosenthal grid that can be used for counting sperm.

5.5. Sperm viability measures

5.5.1. Reagents

- Diluent (10 mM HEPES, 150 mM NaCl, 10% BSA, pH 7.4, see Table 4)
- Invitrogen live/dead sperm staining kit (#L7011)
- Dimethyl sulfoxide (DMSO)

5.5.2. Equipment

- Fluorescent microscope with Rhodamine and FITC filters (for live and dead cells, respectively)
- Dissecting microscope
- Haemocytometer
- Micro-weight balance
- Micro-calipers
- Microdispenser (e.g. Drummond, Fisher Scientific #3-000-225)
- 2 ml glass screw-thread vials and caps (Fisher Scientific #03-391-16)
- Two forceps, dissection tray, and pins
- 1.5 ml microcentrifuge tubes (if you want to save the rest of the queen)
- Water bath set at 36°C

5.5.3. Procedure

1. Make Sybr 14 working solution:
 - a. Add 20 µl Sybr 14 (from Invitrogen kit) to 980 µl DMSO
 - b. Store at -20°C in the dark.
2. Make sufficient volume of sperm diluent of HEPES, NaCl, and BSA (HNB). See Table 4
3. For spermathecae
 - a. Transfer diluent with sperm (from step 5.4.1.) to a labelled 2 ml glass vial.
 - b. After all spermathecae have been dissected, add 10 µl Sybr 14 in DMSO to each vial.
 - c. Incubate 5-10 min at 36°C.
 - d. Add 5 µl propidium iodide.
 - e. Incubate 5-10 min at 36°C.
4. For seminal vesicles
 - a. Gently disrupt seminal membrane to release sperm in diluent.
 - b. Discard seminal membrane and pour sperm solution into a 2 ml glass vial.
 - c. Stain seminal vesicles:
 - i. Add 10 µl Sybr 14/DMSO.
 - ii. Incubate 15 min at 36°C.
 - iii. Add 6 µl propidium iodide.
 - iv. Incubate another 15 min at 36°C.

5. Turn on fluorescent lamp and camera, and open photo software on computer.
6. Use microdispenser to load haemocytometer with 10 µl sperm solution across both chambers.
7. Place haemocytometer on microscope table and examine under low magnification to centre view on grid (Fig. 26).
8. Switch to high magnification (200x then 400x).
9. Turn off visible light and open fluorescence. Focus using FITC filter, then take an image using the camera. This will be a picture of the dead sperm in the field.
10. Without moving the haemocytometer, switch to Rhodamine filter and take another image. This will be a picture of the live sperm in the same field.
11. Move to a new field of view and repeat; a total of five replicates should be minimum, preferably greater particularly if there is large variation among fields.
12. Save pictures using descriptive names indicating sample number, live or dead sperm, and picture number.
13. Switch back to low-resolution and close fluorescent lamp aperture.
14. Clean haemocytometer and cover slip with a kimwipe.
15. Repeat for each new sample.

6. Acknowledgements

The COLOSS (Prevention of honey bee COLony LOSSes) network aims to explain and prevent massive honey bee colony losses. It was funded through the COST Action FA0803. COST (European Cooperation in Science and Technology) is a unique means for European researchers to jointly develop their own ideas and new initiatives across all scientific disciplines through trans-European networking of nationally funded research activities. Based on a pan-European intergovernmental framework for cooperation in science and technology, COST has contributed since its creation more than 40 years ago to closing the gap between science, policy makers and society throughout Europe and beyond. COST is supported by the EU Seventh Framework Programme for research, technological development and demonstration activities (Official Journal L 412, 30 December 2006). The European Science Foundation as implementing agent of COST provides the COST Office through an EC Grant Agreement. The Council of the European Union provides the COST Secretariat. The COLOSS network is now supported by the Ricola Foundation - Nature & Culture.

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