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REVIEW

Matchmakers' Tricky Task: A Comparative Review of Instrumental Insemination in *Apis mellifera* L. and the Associated Challenges in *Apis cerana indica* Fabricius

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Abstract

Instrumental insemination is an important tool in honey bee breeding as it enables controlled mating, genetic improvement, and conservation of valuable traits. This technique is widely practiced in the European honey bee (*Apis mellifera* L.), but its application in the Indian honey bee (*Apis cerana indica* Fabricius) is limited due to species-specific biological, behavioral, and technical challenges. In this review, honey bee biology relevant to instrumental insemination is analyzed, and a comparative assessment of the technique in *A. mellifera* and *A. cerana indica* is presented. Key aspects such as caste differentiation and sex determination, drone congregation areas, natural mating behavior, procedures of instrumental insemination, and factors influencing insemination success, such as queen age, semen collection and handling, carbon dioxide exposure, and post-insemination management, are discussed. In *A. cerana indica*, smaller body size, reduced spermathecal capacity, and increased sensitivity to handling are identified as major constraints affecting insemination efficiency and post-insemination performance. Methodological limitations, procedural standardization, and operator-dependent variability influencing insemination outcomes are also analyzed. Understanding these interspecific differences is essential for refining species-specific protocols and expanding the application of instrumental insemination for selective breeding, genetic conservation, and sustainable apiculture. The review also outlines future research prospects to improve efficiency and promote wider adoption of instrumental insemination, particularly in indigenous honey bee species.

Introduction

Sustainable agricultural production with minimal environmental degradation is essential for achieving food security, as the global population is expected to reach 9-10 billion by 2050 (Van der Sluijs & Vaage, 2016). Pollination is regarded as one of the most essential natural services supporting sustainable crop production (Tirfie & Getachew, 2024). Pollination-dependent crops are five times more valuable than pollination-independent crops, with the global economic crop value ranging between US$235 and US$577 billion annually (Food and Agriculture Organization [FAO], 2018). About 75% of the world's angiosperms are pollinated by honey bees (Wakgari & Yigezu, 2021). Nearly one-third of the total human dietary supply is provided by bee-pollinated crops (Khalifa et al., 2021).

The decline in honey bee populations threatens agricultural production (Grossman, 2013; Wakgari & Yigezu, 2021). The exposure of honey bees to different threats, such as pests and diseases (Pasho et al., 2021; Zapata-Hernández et al., 2024),

pesticide exposure (Tsvetkov et al., 2017; Zapata-Hernández et al., 2024), climate and environmental stresses negatively affects bee development, reproduction, and survival (Khalifa et al., 2021).

Bee breeding is essential to sustain the bee population, increase colony output, and reduce the decline caused by biotic and abiotic stressors. The natural evolution of honey bees is altered by modern beekeeping practices like artificial queen rearing, routine queen replacement, disease treatments, and migratory management. Traits such as resistance to diseases and parasites, efficient food gathering, high queen egg-laying ability, and long colony life can be improved by breeding (Lin et al., 2025).

Effective bee breeding depends on controlled mating, as it enables the selection of both parental lines, leading to reliable genetic progress. Natural mating involves queens mating with numerous unknown drones, creating high genetic variability, uncertain parentage, and limited control over drone contributions (Plate et al., 2019). Control of the drone line is important because drones directly influence worker genetics. Healthy drones improve the transfer of resistance traits, ensuring that selected queens are fertilized by drones from strong and well-adapted colonies (Meixner et al., 2010).

An important tool in the honey bee breeding program is instrumental insemination, as it provides control over both parental lines, which is difficult to achieve under open mating conditions. The introduction of instrumental insemination in 1926 is a milestone in honey bee breeding (Rothenbuhler, 1958). It supports genetic improvement and conservation of valuable or endangered genetic lines (Lin et al., 2025).

Insemination protocols are well established in *Apis mellifera* L., whereas in *Apis cerana* Fabricius, despite their major ecological and apicultural importance for beekeeping across South and Southeast Asia (Oldroyd & Nanork, 2009), the application of instrumental insemination has been limited. The limited application of instrumental insemination is due to species-specific biological and technical limitations in *Apis cerana indica* Fabricius, such as small drone and queen size, low semen yield per drone, heavy mucus contamination of semen during eversion of the drone's endophallus, and reduced sperm storage capacity in the spermatheca (Woyke, 1973, 1975). For effective breeding, genetic improvement, and conservation of *A. cerana indica,* a species-specific instrumental insemination technique is necessary.

Colony structure and development

Honey bees, highly social insects, have a well-developed colony organization comprising three physically and functionally distinct groups: queens, workers, and drones. Eusocial insect colonies generally function as a unified living system in which thousands of insects cooperate to maintain the colony's harmony, growth, and survival. Their well-developed communication systems, which enable the coordination of activities, efficient resource sharing, and regulation of social hierarchies, contribute to their success (Conte & Hefetz, 2008). They sustain their colonies through shared brood care, overlapping generations, and a reproductive division of labor, where a single fertile female coexists with numerous sterile females (Michener, 1974; Kocher & Grozinger, 2011).

Queen

The queen, as the mother of the colony (Yadav et al., 2017), is a single fertile female, and her fitness and reproductive determine the strength and performance of the colony (Metz & Tarpy, 2019). Queen cells are elongated, peanut- or finger-shaped, and the larva that emerges is fed royal jelly, a highly nutritious secretion from worker bees' head glands (Metz & Tarpy, 2019). Adult queen bees are larger, approximately 6–8 mm longer than workers, with a long, pointed abdomen. Their primary role is colony development, and they lack pollen-carrying structures (Yadav et al., 2017). Although the queen is central to reproduction, successful colony propagation also relies on the contributions of the male caste.

Drones

Drones are haploid individuals that develop from unfertilized eggs produced through parthenogenesis. They primarily locate a virgin queen and mate with her during mating flights (Winston, 1991). The quality of the queen and drones influences the colony's health. When a queen mates with drones that have poor reproductive capacity, she is insufficiently inseminated, which ultimately weakens the colony's performance (Metz & Tarpy, 2019). A colony consists of hundreds to thousands of seasonal drones (Brutscher et al., 2019). Drone production in a colony starts three to four weeks before the production of queens. This usually occurs across honey bee colonies because when virgin queens are ready for nuptial flight, sexually matured drones should be available from nearby colonies (Page Jr, 1981; Rangel & Fisher, 2019). Unmated drones live for approximately a month (Metz & Tarpy, 2019) and are evicted from the hives by workers (Free & Williams, 1975). Drones are larger than workers and lack structures for colony work, including pollen baskets and wax-producing glands. Drones cannot feed independently because their tongues are too short to reach the nectar in flowers, and they rely on worker bees for nourishment (Halvacı et al., 2023).

Workers

They are functionally sterile diploid females that perform all colony functions except reproduction. They are genetically similar to queens, and differences in larval nutrition lead to limited ovarian development and to morphological traits suited to colony labor (Evans & Wheeler, 1999). After emergence, workers undertake specialized roles within the colony, such as tending to the queen, rearing the brood, foraging for pollen, constructing wax combs, and performing hygiene maintenance, which are allocated according to the worker's age.

They possess a barbed stinger, and when they sting, the stinger becomes lodged in the target, causing the bee to die shortly after (Yadav et al., 2017**;** Halvacı et al., 2023).

Sex Determination

The life cycle follows holometabolous development, and the duration varies among castes. Queens have the shortest developmental period of 16 days, workers 18-22 days, and drones the longest at 24 days (Yadav et al., 2017). Sex determination is through haplodiploidy combined with single-locus complementary sex determination (sl-CSD), in which males are haploid and develop from unfertilized eggs, and females from fertilized diploid eggs heterozygous at the *csd* locus (Beye et al., 2003). This is governed by the queen's controlled sperm release during egg-laying. The queen's fully functional reproductive organs, including enlarged ovaries and a sperm-storing organ (spermatheca), develop during pupation, whereas workers exhibit undeveloped structures (Gotoh & Sasaki, 2021).

Drone Reproductive Anatomy

The male reproductive system comprises a pair of testes, seminal vesicles, accessory glands, and a complex, eversible endophallus. Spermatogenesis is completed before adult emergence (Rangel & Fisher, 2019**;** Power et al., 2020**;** Klein et al., 2021). One to two weeks after emergence, sperm move from the testes to the seminal vesicle, coinciding with testicular degeneration and functional inactivity (Slater et al., 2021). The seminal vesicle primarily stores sperm, and the amount of sperm directly affects a drone's reproductive quality and ability to compete for mates (Metz & Tarpy, 2021). Sperm viability is enhanced by fluids from the accessory glands that mix with them (Slater et al., 2021). Copulation occurs in flight, and the endophallus everts due to hemolymph pressure generated by contracting abdominal muscles, thereby delivering semen to the female reproductive tract (Özkök & Selcuk, 2020). After copulation, drones die due to endophallus rupture (Brutscher et al., 2019). Mating depends on specialized features for flight, such as large chest muscles, wide wings, and large compound eyes, enabling long flights and accurate queen tracking in drone congregation areas (Halvacı et al., 2023).

Female Reproductive System

The queen's reproductive system primarily supports fertility and utilizes stored sperm throughout her life after a brief mating period (McAfee et al., 2025). The reproductive organs comprise a pair of ovaries containing several hundred ovarioles that mature after mating, enabling her to lay eggs throughout her reproductive life (Jackson et al., 2011; Kozii et al., 2022). Each ovary connects to the lateral oviducts, which join to form a median oviduct leading to the genital/stinger chamber (Halvacı et al., 2023). Sperm is transported to the spermatheca, which is connected to a spermathecal gland and muscular pump (Gotoh & Sasaki, 2021). Together, these structures control sperm entry, release, and long-term storage (Baer et al., 2016; Rangel et al., 2021). The spermatheca provides optimal conditions, like low oxygen, antioxidant protection, and specific nutrients and proteins, enabling sperm to remain viable and functional for several years without further mating (Liu et al., 2020; McAfee et al., 2025).

Drone Maturation

Drone sexual maturation occurs after emergence, and the time can vary due to several factors, including temperature, nutrition, and colony conditions, rather than a fixed maturity period. Previous estimates of the age at which honey bee drones reach sexual maturity vary widely, ranging from 6–8 days to 16 days post-emergence (Rangel & Fisher, 2019). The movement of sperm from the testes to the seminal vesicles and the development of accessory glands mark sexual maturity. Drones remain in the brood during the initial days after emergence. They are fed and groomed by workers before initiating orientation flights that precede mating flights (Goins & Schneider, 2013). Orientation flights typically begin approximately five to eight days post-emergence and allow drones to learn local landmarks and nest location (Rangel & Fisher, 2019). After completing orientation flights, drones start their mating flight to drone congregation areas (DCAs), marking the functional onset of reproductive behavior.

Drone Congregation Areas (DCA)

Drone Congregation Areas (DCAs) are distinct airborne mating locations (Shweta et al., 2025). In *A. mellifera*, DCAs are 30-200 m in diameter (Rangel & Fisher, 2019), where approximately 11,000 drones wait 10-40 m above ground level to mate with the queen (Koeniger et al., 2005b; Rangel & Fisher, 2019). Drones from approximately 240 colonies within a 5 km flight radius are present in DCAs (Rangel & Fisher, 2019). In *A. mellifera*, drones are attracted to the queen and are spread more evenly in the air. In contrast, *A. cerana* differs greatly in its drone congregation areas. In Sri Lanka and Borneo, *A. cerana* drones have been reported to gather near or within treetops rather than in open air, whereas in Japan, *A. cerana japonica* drones gather in open air above trees. These differences between species and regions in congregation behavior are thought to reflect adaptation to local environments, including landscape structure and predation pressure, as reviewed by Koeniger and Koeniger (2000).

The attraction of drones and queens to DCAs is explained by two hypotheses (Shweta et al., 2025). The first is the behavioral DCA hypothesis, in which queens' and drones' behavioral interactions result in DCAs (Koeniger et al., 2005a; Shweta et al., 2025). The second is the physical DCA hypothesis, in which drone orientation is determined by landscape features (Loper & Taylor Jr., 1987). At DCAs, queens release a sex attractant that primarily consists of 9-oxo-trans-2-decenoic acid, attracting all honey bee drones (Brandstaetter et al., 2014). Barriers to breeding between

different species, even though *A. mellifera* and *A. cerana* queens produce similar sex attractants, which are mainly due to differences in mating flight periods (Koeniger, 1988). These features of DCAs and their species-specific variation are illustrated in Fig 1.

Mating Flights

Flight activity and mating behavior vary across species, subspecies, and regions, influenced by differences in heat resistance, flight behavior, and mating patterns. The duration of mating flight in *A. cerana indica* is 27 minutes, and the queen mates with approximately 10 drones during flight (Woyke, 1975). The overall duration of drone and queen flights and their timing in the early afternoon are nearly identical in *A. mellifera* and in geographically separated populations of *A. cerana*, suggesting similar evolutionary pressures on the timing of mating flights (Koeniger & Koeniger, 2000). This exact time separation of mating flights among *Apis* species living in the same area effectively minimizes cross-breeding, preventing interspecific reproduction (Koeniger & Koeniger, 2000). This interspecific and geographic variation in flight schedules is summarized in Table 1, which compares the mating flight timing of queens and drones across different *Apis* species and regions.

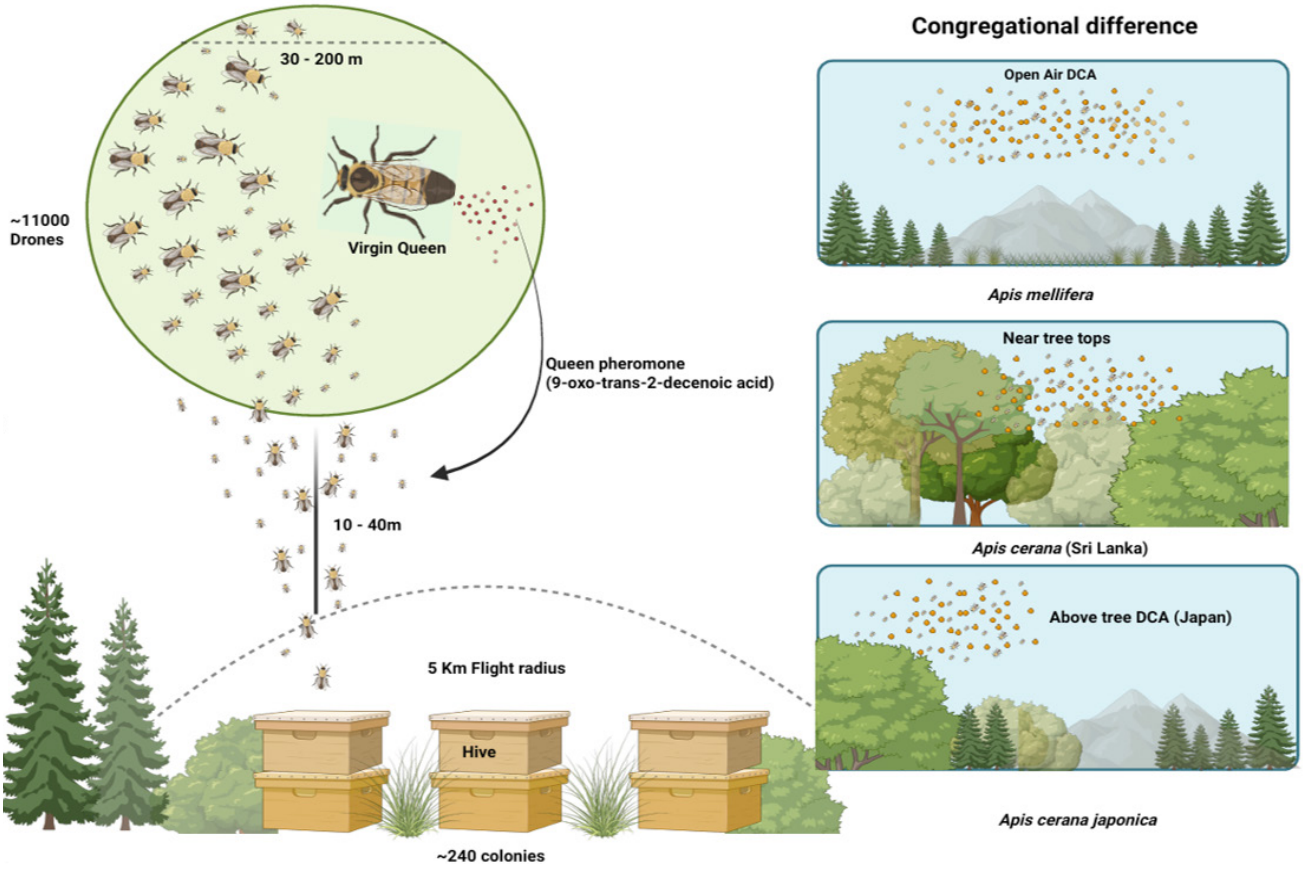

Fig 1. Schematic representation of a drone congregation area (DCA) in honey bees.

Natural Copulation Process in Honey Bees

A large number of drones assemble and follow the virgin queen when they enter the DCAs in a comet-like swarm, engaging in intense competition for mating opportunities. This aggregation is due to the combined effect of smell and sight signals (Brandstaetter et al., 2014; Gries & Koeniger, 1996). Initially, the endophallus everts during mating. In drones of *A. cerana*, it is a fluid-driven process, as abdominal muscles contract, increasing hemolymph pressure and forcing genital structures to evert. First, posterior abdominal sclerites and the penis valve open, followed by the eversion of the vestibulum up to the hairy dorsal triangular plate, then bursal cornua and three pairs of upper cornua, which evert in a different sequence from *A. mellifera*. The most forceful stage is the cervix eversion, after which the bulb of the endophallus lies within the cervix and semen is expelled together with glandular secretions under continued pressure. Further pressure leads to eversion of the fimbriate lobe and, in *A. cerana*, the formation of a hemolymph-filled vesicle at the terminal bulb due to separation of tissue layers, a phenomenon not observed in *A. mellifera*; complete eversion ultimately facilitates semen transfer to the queen's reproductive tract and contributes to pair separation after mating (Ruttner et al., 1973).

Table 1. Mating flight timing in queens and drones shows broad variation across geographic regions and subspecies.

S. No	Species/Subspecies	Caste	Type of Flight	Time Period (h)	Location	Features	Reference
1	*Apis cerana indica*	Drone	General flight	10:30–15:00	Germany	Early start; peak at 12:00–14:00 h; weak temperature dependence	(Ruttner et al., 1972)
2	*Apis cerana cerana*	Drone	General flight	>16:00	Germany (Peking strain)	Late-afternoon flights observed	(Ruttner et al., 1972)
3	*Apis cerana indica*	Queen	Mating flight	13:30-15:30 (mating mainly 14:00-15:00 h)	Mahabaleshwar, Maharashtra, India	Peak flights at 14:15–14:30 h; mean duration 27 min	(Woyke, 1975)
4	*Apis cerana indica*	Queen	Mating flight	12:40–13:40	India	Earlier mating period	(Verma, 1991)
5	*Apis cerana indica*	Queen	Mating flight	13:30-14:30 h	Graswang valley, Germany	Flights with mating sign lasted 17–46 min (avg. 30.8 min)	(Ruttner et al., 1972)
6	*Apis cerana indica*	Drone	Mating flight	15:30-17:00h	Sri Lanka	Late-afternoon mating	(Punchihewa et al., 1990)
7	*Apis cerana indica*	Drone	Mating flight	13:25–14:55	India	Early-afternoon peak	(Verma, 1991)
8	*Apis cerana indica*	Drone	Mating flight	16:15-17:15h more activity at 17:00h	Sri Lanka	Late mating flights	(Koeniger & Wijayagunasekera, 1976)
9	*Apis cerana japonica*	Queen	Mating flight	13:15-17:00h	Japan	Later and longer flights	(Yoshida et al., 1994)
10	*Apis cerana japonica*	Drone	Mating flight	13:15-16:30h	Japan	Extended overlap with queen flights	(Yoshida et al., 1994)
11	*Apis mellifera*	Queen	Mating flight	12:15-15:00h	Japan	Earlier flight than *Apis. cerana japonica*	(Yoshida et al., 1994)
12	*Apis mellifera*	Drone	Mating flight	11:30-15:00h	Japan	Drones fly ~1.5–2 h earlier	(Yoshida et al., 1994)

Successful copulation results in the sudden death of the drone, whereas queens continue to mate with multiple drones during a single mating period (Brandstaetter et al., 2014)**.** The hemolymph pressure required to evert the endophallus and the forceful ejaculation of semen cause the death of the drone, making mating possible only once (Woyke, 2008**;** Goins & Schneider, 2013). The complete eversion of the endophallus leads to ejaculation during copulation (Woyke, 2008). Semen is released into the female reproductive tract. After copulation, the distal slender portion of the drone's endophallus ruptures and remains in the queen's sting chamber, known as the "mating sign" (Koeniger, 1990; Woyke, 2008). The mating sign is a remnant, but it also contributes to behavior, as queens with a mating sign are more attractive to subsequent drones than queens without one (Rangel & Fisher, 2019). This reflects cooperative drone behavior (Rangel & Fisher, 2019), in which they communicate about the accessibility of the receptive queen for mating (Koeniger, 1990). This signaling may potentially help in subsequent matings during a single nuptial flight, thereby contributing to mating success and polyandry.

Polyandry

Polyandry, mating with multiple males, is a feature of honey bee queens' reproduction in the genus *Apis* (Palmer & Oldroyd, 2000; Roberts et al., 2015). Genetic analysis of worker bees showed that queens mate with multiple drones, resulting in high genetic variety within the colony (Schlüns et al., 2005). Queens of *A. mellifera* mate with an average of 13-20 drones, with values ranging from 8-32 (Lago et al., 2023). Queens of *A. cerana* mated with an average of 27.4 males (range 16-42) in the invasive Australian population and 29.0 males (range 19–46) in native Chinese populations (Ding et al., 2017).

Early studies documented that queens may make multiple mating flights when mating success during earlier flights is low (Roberts, 1944). *A. mellifera* queens, when restricted to a single flight, had fewer matings than unrestricted queens, even though egg-laying was artificially stimulated, indicating repeated flights increase mating opportunity (Schlüns et al., 2005). Polyandry is strongly linked to queen reproductive success in *A. mellifera,* as mating frequency is positively associated with sperm stored in the spermatheca. Queens mating with more drones store higher sperm quantities, thereby ensuring a lifetime supply for egg fertilization (Schlüns et al., 2005). Schlüns et al. (2005) demonstrated a nonlinear relationship between estimated mating number and sperm count in the sperm storage organ in naturally mated queens, with sperm storage approaching an asymptotic maximum of approximately three million sperm per queen. Although drones produce millions of sperm, natural mating results in inefficient sperm transfer. After multiple matings, semen is stored in the egg tubes of the queen. Only a small portion of sperm, approximately 5-10%, reaches the spermatheca

(Woyke, 1962). This inefficiency explains why multiple matings are important to ensure adequate sperm storage (Kraus et al., 2004). Instrumental insemination studies further support this relationship, showing that multiple inseminations result in higher spermathecal sperm counts than single inseminations (Bolten & Harbo, 1982). Thus, instrumental insemination has been adopted as a practical method to mimic natural polyandry and ensure adequate sperm storage in breeding programs.

Instrumental Insemination

Instrumental insemination is a crucial tool in bee breeding and research, as it enables precise control over mating by selecting the parental lines (Shweta et al., 2025). The instrumental insemination technique for honey bees began to develop in the early twentieth century, particularly during the 1920s (Cobey, 2007). The queen bees, during their nuptial flight, mate with drones of unknown genetic lineage in mid-air at the drone congregation area, as they are polyandrous in nature (Khan et al., 2022). This procedure achieves control over the paternal lineage, enabling researchers and beekeepers to manage inherited characteristics precisely, and successful insemination and fertilization can improve queens' reproductive performance and colony output (Maucourt et al., 2023; Mansour et al., 2025). In instrumental insemination, queen bees are inseminated with a precise amount of semen using proper equipment under sterile conditions (Khan et al., 2022; Mansour et al., 2025). This involves a series of steps, including preparing drones, collecting semen, immobilizing the queen, insemination, and care after insemination. The success of the procedure depends on handling conditions, including temperature and tissue hydration, to maintain sperm viability and prevent damage to the queen's reproductive tract (Cobey et al.,2013; Cobey, 2016). The sequence of steps involved in instrumental insemination of honey bees is summarized in Fig 2.

Procedure

Selection and Preparation of Drones and Queens

Drones that attain sexual maturity are used for semen collection, and the quality and quantity of semen depend primarily on drone age. Information on drone maturity and semen requirements has been reported separately for *A. mellifera* and *A. cerana indica*. In *A. mellifera*, drones generally attain sexual maturity between 12–14 days after emergence (Cobey, 2016), although maturation may occur as early as 9–12 days depending on environmental conditions (Klein et al., 2021). Instrumental insemination in *A. mellifera* generally requires semen collected from approximately 8 drones (Woyke, 1973).

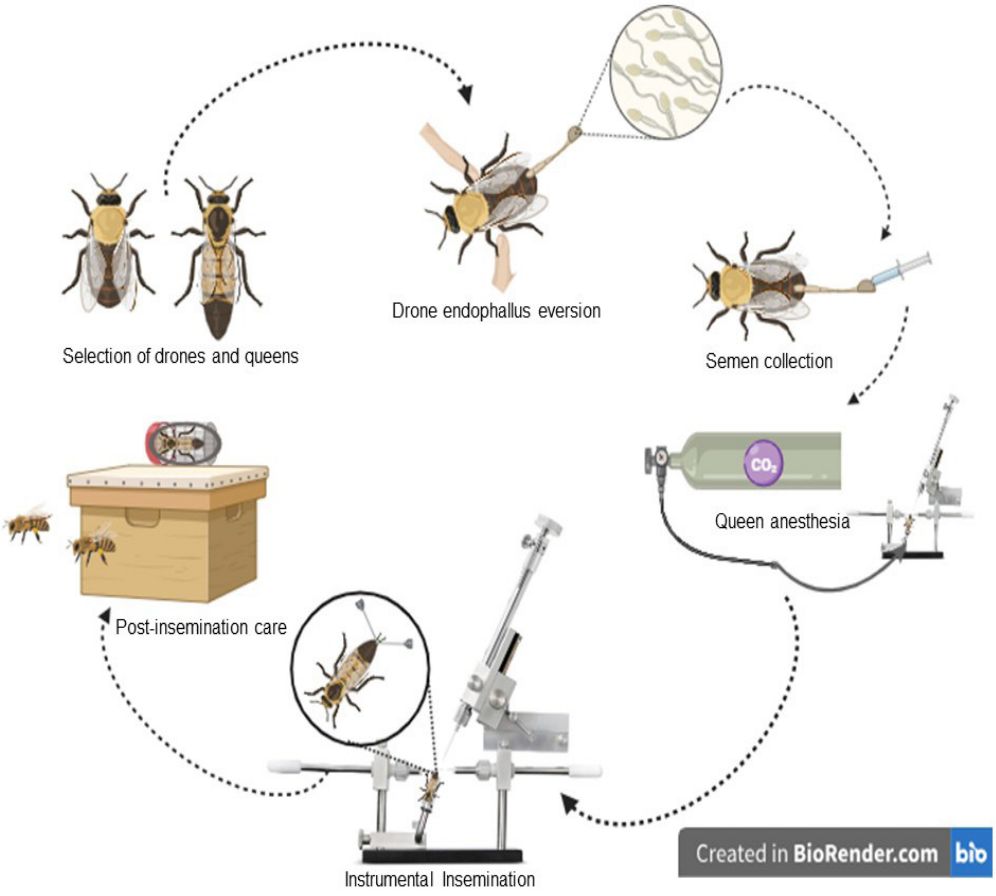

Fig 2. Stepwise schematic of the instrumental insemination process in a honey bee queen.

In *A. cerana indica*, drones also attain sexual maturity approximately 12–14 days after emergence. Sawarkar and Tembhare (2014) reported maximal seminal vesicle development in 12-day-old drones, whereas Borah et al. (2024) used 14-day-old mature drones for instrumental insemination studies. Due to the comparatively lower semen volume produced by *A. cerana indica* drones, semen from about 40–60 drones may be required for instrumental insemination (Woyke, 1973).

Capped drone brood from selected queens is often transferred to nurse colonies to increase the number of drones produced from the preferred genetic line (Büchler et al., 2013). Mature drones can be collected either on the day of insemination or on the previous day**,** commonly by capturing drones returning from unsuccessful mating flights or by removing them from the outer combs of colonies (Cobey et al., 2013).

Queens required for controlled mating are maintained in nurse colonies, which contain large numbers of young worker bees responsible for feeding, grooming, and caring for developing queens (Kama & Shpigler, 2025). The quality of a honey bee queen is strongly influenced by the strength of the nurse colony and the nutritional environment provided by nurse bees, with well-fed, and abundant colonies producing heavier, and more viable queens (Kama & Shpigler, 2025).

The age of the adult queen is a critical factor influencing insemination success. In *A. mellifera*, virgin queens aged 5-12 days after emergence are generally preferred for instrumental insemination (Cobey et al., 2013; Cobey, 2016), and queens inseminated at 7-10 days of age showed better post-insemination performance (Bieńkowska et al., 2008). In *A. cerana indica*, virgin queens older than 5 days, typically 5-12 days after emergence, are commonly used for instrumental insemination (Woyke, 1973; Borah et al., 2024).

Drone Endophallus Eversion

To collect semen from drones, the endophallus is turned inside out, a process that occurs in two sequential steps: partial eversion and complete eversion (Cobey, 2016; Khan et al., 2022; Borah et al., 2024). During partial eversion, the drones are held vertically, and pressure is applied with thumb and forefinger; the abdominal muscles contract, exposing the cornua (Mackenson & Tucker, 1970; Cobey, 2016). The cornua are paired, horn-like structures of the endophallus that help in stabilizing the drone during mating (Woyke, 2008). Cobey et al. (2013) assessed drone sexual maturity by cornua coloration. Sexually immature drones possess uncolored cornua, whereas mature drones have orange-pigmented cornua.

In the second step, complete eversion is achieved by applying pressure from the anterior toward the base of the abdomen using the thumb and forefinger, resulting in full eversion of the endophallus and exposure of semen (Mackenson & Tucker, 1970; Cobey, 2016) The drone is held in a downward position to prevent contact between the everted endophallus and the fingers, thereby minimizing contamination (Cobey et al., 2013). In sexually mature drones, semen appears as a creamy, marbled tan mass over a layer of white mucus, a characteristic feature confirming semen suitability before instrumental insemination (Cobey, 2016). Drone defecation during handling is a major contamination source (Stoian et al., 2018).

Semen Collection

A glass capillary tube attached to an insemination syringe is used for semen collection (Cobey et al., 2013; Yániz et al., 2020). The syringe is prepared so that a column of sterile saline solution is within the tip, separated from the semen by the syringe mechanism, allowing controlled movement of fluids during collection and injection (Mackenson & Tucker, 1970; Cobey, 2016). Semen is gently skimmed and drawn into a capillary tip, avoiding the viscous mucous layer, as it will block the tip and can cause difficulties in the insemination process (Mackenson & Tucker, 1970; Cobey, 2016).

Semen collection continues until the desired volume is obtained, as it varies among individual drones (Yániz et al., 2020). Semen from successive drones is collected by releasing the previous semen load into the fresh ejaculate and aspirating it together as a continuous column (Cobey et al., 2013). Throughout the procedure, the capillary tip is kept moist with saline to minimize exposure and protect semen quality (Cobey et al., 2013). Following semen collection, the syringe and components are disinfected with 70% alcohol and thoroughly rinsed with distilled water to maintain aseptic conditions (Mackenson & Tucker, 1970; Cobey, 2016).

Queen Anesthesia

The queen bees are sedated before insemination so that they remain immobilized during the insemination process and can trigger egg-laying (Özkök & Selcuk, 2020). Carbon dioxide is used as an anesthetic during the insemination process (Cobey, 2016). The carbon dioxide cylinder is adjusted to about 5 psi, and the gas flow to the queen holder is reduced to a very gentle stream, just enough to keep the queen calm. The flow is commonly adjusted by dipping the stopper in water and setting it to release about 2–3 bubbles per second (Borah et al., 2024). Two doses are given, the first being given one or two days before the procedure for 1 to 4 minutes, with queens caged individually and placed in carbon dioxide-filled plastic bags or jars, and the second is given during the procedure (Cobey et al., 2013).

Gabka et al. (2022) found that queens receiving a second dose of CO_2 from a few hours to a couple of days after the first dose laid eggs sooner than those that received only one treatment. However, if they receive a second dose too quickly, it slows down egg-laying in queens (Gąbka et al., 2022). Combining nitrogen with CO_2 increases worker attraction to the queen, and nitrogen itself is considered physiologically neutral in bees (Chuda-Mickiewicz et al., 2012).

Insemination of Queen

During insemination, the queen holder and syringe are aligned on the insemination stand at an angle of 30°–45°, which allows the smooth passage beyond the valve fold (Cobey et al., 2013**;** Borah et al., 2024). The vaginal orifice is exposed using a sting hook and a ventral hook by separating the abdominal plates (Cobey, 2016). The syringe tip is then placed above the dorsal margin of the vaginal opening and inserted slightly (about 0.5–1 mm). 8-12 µl per queen is considered to be the standard dose (Cobey et al., 2013). It is then advanced further to bypass the valve fold and reach the median oviduct, allowing semen to be delivered smoothly without damaging the tissues. After semen deposition, the syringe tip is withdrawn carefully, and an air space followed by a saline drop is aspirated into the syringe to prepare for subsequent inseminations when required (Borah et al., 2024).

Post-Insemination Care

Post-insemination, queens are placed in queen cages or push-in cages and then returned to nursery or nucleus colonies (Cobey et al., 2013**;** Borah et al., 2024). Insemination is successful when sperm migrates from the oviducts to the spermatheca, achieved by keeping queens in controlled conditions for approximately two days post-insemination (Cobey et al., 2013; Cobey, 2016). Queens are released into the nucleus colony upon resumption of normal activities (Borah et al., 2024). Improper management of queens can negatively affect queen survival, egg-laying, sperm storage, and colony acceptance, making careful post-insemination handling essential (Cobey et al., 2013; Gąbka & Cobey, 2018). Queens receiving proper post-insemination care exhibit normal reproductive function and colony performance, highlighting the importance of quality post-care (Güler et al., 2022). Long-term breeding studies demonstrated that standardized post-insemination management enables the full expression of genetic gains from controlled mating (Maucourt et al., 2023). Imaging-based studies of the queen reproductive system confirmed sperm distribution and retention continue post-insemination, reinforcing the importance of a calm recovery time for successful sperm storage and oviposition initiation (Dziechciarz et al., 2025).

Post-Insemination Evaluation

Dissection of the spermatheca is a post-insemination evaluation method to verify insemination success in honey bee queens. It assesses sperm migration, storage efficiency, and sperm viability within the queen's reproductive system (Cobey et al., 2013). After insemination, the semen is deposited in the lateral oviducts, and it takes approximately 24-40 hrs for the spermatozoa to migrate into the spermatheca (Woyke, 1983). Dissecting the spermatheca after this period confirms the successful sperm transfer and long-term storage (Cobey et al., 2013). Although age-related declines in spermathecal sperm viability have been documented, viability generally remains sufficiently high in older queens to support normal fertilization over extended periods, underscoring the spermatheca's role as a stable long-term storage organ and validating its use as a benchmark for insemination success (Lodesani et al., 2004**;** Kahya & Gençer, 2022).

Queens that are properly inseminated have a creamy tan spermatheca with a marbled swirl pattern; poorly mated queens have a cloudy spermatheca, whereas virgin queens have a clear spermatheca (Cobey et al., 2013). Spermathecal dissection permits genetic and molecular analyses, such as identifying drone parentage and mating structure using microsatellite markers or high-resolution melting analysis, which are useful for research and breeding studies (Kahya, 2020; Moškrič et al., 2023). Spermathecal dissection is a destructive technique, so its use is generally restricted to research and validation, whereas in practical breeding and commercial operations, insemination success is judged indirectly through queen survival, onset of egg laying, brood quality, and overall colony performance (Cobey et al., 2013). The success of instrumental insemination is influenced by several biological, technical, and environmental factors that affect sperm viability, queen acceptance, and subsequent colony performance.

Key Factors Affecting the Performance of Instrumentally Inseminated Queens

Queen and Drone Rearing Conditions

The performance of inseminated queens mainly depends on the quality of the queens and drones. Queen quality is linked to larval grafting age and worker bee nutrition, directly influencing adult queen size, ovary development, sperm storage capacity, and egg-laying potential (Büchler et al., 2013). Studies have shown that queens from older larvae have lower body weight, fewer ovarioles, and lower sperm storage capacity (Woyke, 1971). Queen weight also indicates quality (Nelson, 1989**;** Cobey, 2007), as heavier queens may sometimes start laying eggs later than lighter queens (Skowronek et al., 2002).

However, drone quality is also critical, as semen quantity and viability vary across drones (Rhodes et al., 2011). Drones reared in the presence of certain miticides may produce sperm that remain viable during mating but decline quickly, potentially dying within six weeks post-mating (Burley et al., 2008; Pettis et al., 2016).

Queen and Drone Age

The virgin queen has a narrow range of sexual maturity (Cobey, 2007). Queens inseminated between 5 and 10 days after emergence consistently store sufficient sperm, whereas queens inseminated too early or too late store fewer sperm and have shorter lifespans (Woyke & Jasinski, 1976). Cobey (2016) stated that insemination of over-aged queens is a major contributor to poor colony performance. Seasonal effects further influence mating age results, with spring-reared queens mating and initiating oviposition more efficiently than late-

season queens (Cobey, 2007). Proper synchronization of queen emergence, sexual maturity, and insemination timing is therefore essential for reproductive success.

Drones become ready to mate approximately 14 days after emergence, with peak reproduction at around 21 days (Rousseau et al., 2015). Colonies eliminate drones under stress, thereby affecting semen availability (Cobey, 2016). The age of the drones is crucial, as sexually immature drones cannot ejaculate semen (Halvacı et al., 2023). When drones age beyond their optimal window, the semen becomes increasingly thick and changes color from yellowish-cream to orange-brown, complicating collection efforts for instrumental insemination (Smilga-Spalvina et al., 2023). Furthermore, the seasonal nature of drone production and the high attrition rates due to environmental stressors require careful planning to ensure a plentiful supply of mature drones for successful instrumental insemination (Cobey, 2007; Smilga-Spalvina et al., 2023).

Skill of Inseminators

The technical skill of an inseminator is a key factor in achieving successful insemination. The insemination process requires precise manipulation, including successfully bypassing the vaginal valve fold, precise semen deposition into the lateral oviducts, and avoidance of mechanical injury to the queen (Cobey, 2016). Any errors can lead to low sperm migration, queen mortality, or failure of insemination procedures. Precision and accuracy of the inseminator play a major role in the success of the procedure (Khan et al., 2022).

Semen Quantity, Quality, Handling, and Storage

Short-term storage of semen is feasible. It can be stored for up to two weeks at 13 °C with good viability (Cobey et al., 2013). For long-term storage by cryopreservation, cryoprotectants such as dimethyl sulfoxide (DMSO) under controlled cooling protocols can preserve high levels of sperm viability. Insemination with cryopreserved semen has frequently resulted in abnormal brood patterns and increased production of drones (Cobey, 2007; Hopkins & Herr, 2010). Although short-term semen storage is feasible, long-term cryopreservation remains challenging due to inconsistent fertilization success and colony performance (Hopkins & Herr, 2010).

Standardized protocols recommend a semen volume of 8-12 µl, given in multiple small doses rather than a single dose (Cobey et al., 2013). When a large semen volume is given, sperm migration is slow compared to smaller doses, where faster spermathecal filling is achieved (Cobey, 2007), but the risk of stress and injury is higher.

Semen handling and storage influence sperm viability and the performance of instrumentally inseminated queen bees. The number of spermatozoa entering the spermatheca decreased when a large volume of diluted semen (16 µl) was injected, and 1:1 dilution of 8 µl fresh semen with saline resulted in fewer spermatozoa than undiluted semen (Kahya & Gençer, 2022). The addition of a saline solution to semen did not significantly affect the number of sperm in the spermatheca or the condition of the oviducts (Gąbka & Cobey, 2018).

Sperm viability is affected by improper handling, temperature, air, or light fluctuations (Collins, 2000). Factors such as pH, osmolarity, and diluent composition are crucial for maintaining sperm integrity, and deviations from optimal conditions may cause irreversible damage (Collins, 2000; Hopkins & Herr, 2010).

Spermathecal Filling and Sperm Storage

Spermathecal filling is another factor that determines queen longevity, reproductive success and colony performance. The spermatheca is a specialized organ characterized by a dense tracheal network and a unique chemical environment rich in proteins, sugars, and antioxidants supporting long-term sperm survival (Phiancharoen et al., 2004). Sperm migration from the lateral oviducts to the spermatheca takes approximately 40 hours and depends on the pump and biochemical environment in the reproductive tract (Cobey et al., 2013). Semen quantity affects spermathecal filling and long-term availability; insufficient semen doses lead to reduced sperm storage, queen failure, or early queen replacement (Cobey, 2007). Queens confined in cages stored significantly fewer spermatozoa (approximately 1.27×10^6) than queens maintained in mini-nuclei or full colonies, whose sperm counts in the spermatheca (approximately 2.7×10^6) were comparable to naturally mated queens (Vung et al., 2018). These findings demonstrate that unrestricted movement and normal colony integration after insemination are critical for effective sperm movement and storage.

Pre and Post-Insemination Queen Management

After insemination, queens are banked in nursery colonies for 48 hours to allow sperm to move before being introduced to nucleus colonies (Cobey, 2016). Variable pheromonal changes are exhibited by instrumentally inseminated queens, requiring more careful introduction, as inadequate banking can lead to queen rejection or early supersedure (Cobey, 2007). Queens maintained at brood-nest temperature and attended by a sufficient worker population exhibit efficient sperm migration and earlier oviposition, whereas small-nucleus colonies with fewer workers are associated with delayed oviposition and incomplete clearing of semen from the oviducts (Woyke & Jasiński, 1990). Studies confirmed that queens with attendant workers store more spermatozoa than queens banked without workers, highlighting the importance of worker contact during the critical post-insemination period (Gąbka & Cobey, 2018).

Temperature during Queen Storage

Temperature is another environmental factor affecting the success of instrumental insemination. Conditions close to brood-nest temperature (≈34 °C) after insemination promote efficient sperm migration from the oviducts to the spermatheca, compared to lower temperatures (Woyke & Jasinski, 1976).

For sperm migration to the spermatheca, temperature acts as a critical factor. Most sperm migration occurs within the first hours after insemination, and temperature, hive conditions, and colony size can either enhance or inhibit this process (Buescu et al., 2015).

Carbon Dioxide (CO_2)

Carbon dioxide has a positive effect on queen performance (Cobey, 2007). Moderate CO_2 exposure before and during insemination has been shown to enhance sperm migration, accelerate the onset of oviposition, and reduce the chance of unwanted mating flights (Fischer, 1990; Woyke et al., 2001). If the CO_2 concentration exceeds 80% and is maintained for more than 3 minutes, it can be harmful. To reduce harmful effects, N2 gas is used in moderation alongside CO_2 (Czekońska, 2009).

Pheromone

Pheromones play diverse roles in the honey bee colony, including aggregation, mate attraction, and the regulation of queen and worker behavior. The production and composition of these chemical signals can be influenced by factors associated with instrumental insemination, such as CO_2 exposure, semen quality and quantity, and the skill of the inseminator in manipulating the queen's genital tract (Cobey, 2016). Virgin queens produce pheromonal blends that differ markedly from those of mated queens, reflecting changes associated with reproductive status. In queens, pheromones are primarily synthesized in the mandibular and dufour's glands (Khan et al., 2022). Queen mandibular pheromones (QMPs) exert both short-term and long-term effects on worker behavior and physiology (Richard et al., 2007).

Sanitation

Sanitary conditions are critical to the success of instrumental insemination in honey bee queens, as injury and infection are among the major causes of failure (Cobey, 2016). Drones often defecate during semen collection, and the exposed endophallus can contaminate the semen; hence, cleanliness is necessary during handling to avoid the transmission of microbes and mechanical obstruction of the syringe tip (Cobey, 2016). Common guidelines focus on sterilizing all components of instruments (with heat or alcohol), sterile vials and capillaries, bacteriologically filtered or heat-sterilized diluents, and routine disinfection of equipment and workstations so that contamination is minimized (Cobey et al., 2013). Cobey (2016) also observed that poor outcomes are typically associated with inefficient procedures and hygiene, whereas good technique and sanitation give queens that perform as well as those that are naturally mated. Together, these results point to sanitation as a serious factor affecting sperm viability, queen health, and general insemination success. Despite the availability of standardized instrumental insemination protocols, their direct application across different *Apis* species is limited due to major anatomical and physiological differences.

Comparative Analysis: *A. mellifera* vs *A. cerana indica* in Instrumental Insemination

The effectiveness of instrumental insemination in honey bees is related largely to the physiology of the drone and the queen (body size, the size of reproductive organs, semen, and mating physiology) which determine success. Such biological and behavioral variations between species like *A. mellifera* and *A. cerana,* especially *A. cerana indica* have some limitations to the use and standardization of the process. The first model for which these methods and procedures of instrumental insemination were set was for *A. mellifera*. Adult *A. mellifera* testes and ovaries are larger than those of *A. cerana indica* (Kapil, 1962); therefore, *A. cerana indica* produce fewer sperm than *A. mellifera.*

Semen Yield per Drone and Consequent Drone Requirements for Insemination

In a study by Ruttner and Maul (1969), reported that *A. cerana indica* drones produce approximately 1.0-1.5 million spermatozoa per drone, which is only 10-15% of the sperm produced by *A. mellifera* drones (Ruttner et al., 1972). According to Vung et al. (2016), it is estimated that 17 drones are killed and 11.94 drones discharge their semen successfully to obtain 1µl of semen in *A. cerana*, and 0.09-0.1uL of semen is produced per drone on average. Research carried out by Woyke (1975) found that *A. mellifera* produces 7.5 times more semen and 11 times more spermatozoa as compared to *A. cerana indica* (Woyke, 1975). The volume of semen produced is low in *A. cerana indica*, and thus, additional drones are needed to carry out the insemination procedure. To achieve efficient insemination, the *A. cerana indica* queen requires about 40-60 drones, for which 100-150 are killed. In contrast, *A. mellifera*, 8 drones are needed, for which 16-24 are killed (Woyke, 1973). This necessitates the use of many drones, which complicates and intensifies the insemination process in *A. cerana indica*.

Endophallus Morphology and Its Influence on Semen Collection Efficiency

The other factor is the endophallus nature of the species. In *A.cerana indica*, the endophallus lacks chitinized plates and is a soft, flexible cornua covered by a thick orange adhesive mucus layer that envelops the ejaculate (Ruttner et al., 1973). The absence of these plates reduces mechanical resistance during eversion; thus, the process will proceed fast, leaving only a very brief stage in which semen can be accessed before it becomes mixed with mucus, making semen collection for this process difficult (Ruttner et al., 1973). In *A. mellifera*, these chitinized plates provide support and resistance, producing a gradual and staged eversion that facilitates controlled semen collection.

Mucus Secretion and Mechanical Constraints During Semen Handling

A.cerana indica produces a lot of mucus upon endophallos eversion and, therefore, it is hard to isolate semen, and the amount of usable ejaculate is low (Woyke, 1973). This mucus clogs insemination syringes, thus adding another technical constraint (Woyke, 1973) . The higher sperm count, less viscous semen, and structural adaptations of the reproductive organs of *A. mellifera* help cleaner semen handling and successful standardization of instrumental insemination protocols.

Limited Usable Semen, Seasonal Constraints, and Drone Availability

Only about one-third of drones yield usable semen, with a mean post-eversion volume of approximately 0.16 mm^3 per drone, highlighting the need for semen pooling from many drones to achieve effective insemination (Woyke, 1973). A narrow period is available for semen collection in *A.cerana indica,* as strong seasonal and environmental constraints impact drone production and flight activity (Woyke, 1975). As a result, the availability of sexually mature drones suitable for semen collection is limited in time.

Species-Specific Queen Responses to Insemination and Sperm Utilisation

The difference in the queen's response to insemination also has a strong disparity between the two species. In *A. mellifera*, standardized insemination techniques developed through long-term experimental refinement resulted in predictable sperm migration to the spermatheca and reliable onset of oviposition, forming the basis for reproducible breeding protocols (Ruttner et al., 1973; Woyke, 1973). In *A. cerana indica*, however, large single insemination doses failed to empty their oviduct fully, indicating that mucus viscosity interferes with sperm transport to the spermatheca; although repeated inseminations with smaller volumes improve outcomes, indicating that queen acceptance and sperm utilization are strongly influenced by species-specific reproductive tract physiology (Woyke, 1973).

There are well-established and standardized protocols for instrumental insemination in *A. mellifera.* The biological advantages, along with abundant drone availability, manageable semen viscosity, and robust queen morphology, helped in refining insemination instruments and procedures that are now widely adopted in breeding and research programs. Later methodological syntheses further optimized *A. mellifera* insemination by standardising semen pooling, temperature control, queen handling, and post-insemination care, resulting in consistently high success rates and long-term queen performance (Cobey et al., 2013). The key biological and technical differences between the two species that influence instrumental insemination are summarized in Table 2. The differences summarized in Table 2 emphasize the need for species-specific modifications and technological innovations to improve instrumental insemination success, particularly in *A. cerana indica.*

Table 2. Comparative biological and technical constraints affecting instrumental insemination in *Apis mellifera* and *Apis cerana indica*.

Parameter	*Apis mellifera*	*Apis cerana indica*	References
Number of ovarioles	Average of about 300 ovarioles (maximum up to 400)	Average of about 200 ovarioles	(Hepburn & Radloff, 2011; Pan et al., 2025)
Egg laying capacity	1500–2000 eggs per day	400–600 eggs per day	(Hepburn & Radloff, 2011; Pan et al., 2025)
Number of testiolar tubules per testis	150–200	7	(Lago et al., 2020; Sawarkar & Tembhare, 2015)
Total testiolar tubules per drone	300–400	14	(Lago et al., 2020; Sawarkar & Tembhare, 2015)
Sperm per drone	~10–12 million spermatozoa	1.0–1.5 million spermatozoa (10–15% of *A. mellifera*)	(Ruttner et al., 1972)
Drones required per queen for successful instrumental insemination	8 drones (16–24 killed)	40–60 drones (100–150 killed)	(Woyke, 1973)
Endophallus structure	Chitinised plates present, rigid	Soft, flexible, lacks chitinised plates	(Ruttner et al., 1973)
Endophallus eversion	Slow, staged, controllable	Very rapid, brief accessible stage	(Ruttner et al., 1973)
Mucus production	Low	Very high, orange adhesive mucus	(Ruttner et al., 1973)
Ease of semen collection	Easy, clean ejaculate	Difficult, semen mixes with mucus	(Ruttner et al., 1973; Woyke, 1973)
Syringe clogging	Rare	Frequent due to mucus	(Woyke, 1973)
Usable ejaculates	High proportion	Only ~1/3 drones yield usable semen	(Woyke, 1973)
Drone availability	Long breeding season	Narrow seasonal window	(Woyke, 1975)
Queen response to insemination	Predictable sperm migration and oviposition	Large doses fail; repeated small doses needed	(Woyke, 1973)
Standardised protocols	Well-developed and reproducible	Not fully standardised	(Cobey et al., 2013)

Future Prospects

Prospects for Improving Instrumental Insemination in *A. cerana indica*

A high level of standardization has been achieved for instrumental insemination in *A. mellifera*, whereas comparable standardization has not yet been attained in *A. cerana indica* due to several biological, physiological, and technical limitations.

The reproductive biology and insemination requirements of *A. mellifera* are comparatively better understood, and most currently available instrumental insemination protocols and devices have been developed specifically for this species (Cobey et al., 2013). Consequently, direct adaptation of *A. mellifera*-based protocols to *A. cerana indica* often results in inconsistent insemination outcome. Compared with *A. mellifera*, *A. cerana indica* exhibits lower semen volume, excessive mucus secretion, high semen viscosity, and smaller queen size, all of which complicate semen collection and insemination procedures (Ruttner et al., 1973). Therefore, future research should focus on developing standardized species-specific insemination protocols tailored to the reproductive physiology and anatomical characteristics of *A. cerana indica*

A major area for improvement is the development of species-specific instrumentation for *A. cerana indica*. Most currently available insemination device, syringe, and tips have been designed primarily for *A. mellifera*, which may reduce semen recovery and increase the risk of mechanical injury in the smaller queens of *A. cerana indica*. In addition, severe mucus contamination of semen in *A. cerana indica* (Woyke, 1973) complicates semen separation and frequently obstructs insemination syringes. Future technological developments may therefore focus on designing ultra-fine insemination syringe tips and micro-capillary syringes with minimal residual dead space and a narrow diameter to facilitate more efficient semen recovery from mucus-contaminated ejaculates and enable precise low-volume semen deposition into the narrower oviducts of *A. cerana indica* queens. Also, redesigning queen holders and ventral and vaginal hooks, suitable for the small size and delicate anatomy of *A. cerana indica* queens, may help reduce tissue injury and improve insemination efficiency.

Optimization of semen dose is an important aspect of protocol standardization. Existing studies have shown that queens of *A. cerana indica,* inseminated with a single large dose of 4 µL, failed to empty their oviducts and showed poor sperm migration, whereas repeated low-volume inseminations partially compensated for these effects (Woyke, 1973). Future protocols can be refined so that they favor multiple low-volume inseminations without causing mechanical injury to the reproductive tissues.

Mucus management represents a potential area for improvement in instrumental insemination of *A. cerana indica*. Woyke (1975) reported that *A. cerana indica* drones produce a thick mucus layer that can interfere with insemination procedures. Studies on this species have shown that mucus gland secretions contain proteins, carbohydrates, and lipids, indicating a complex biochemical composition (Sawarkar & Tembhare, 2010). The mucus is also known to contribute to mating sign formation and sperm transfer processes. Therefore, further studies on the biochemical composition and functional role of mucus may help develop improved semen handling and separation techniques for instrumental insemination without adversely affecting sperm viability or queen performance.

There is considerable scope for improving post-insemination queen management in *A. cerana indica*. Optimized post-insemination handling, such as refined CO_2 treatment regimes, controlled temperature and humidity, and extended recovery periods prior to colony introduction, can improve insemination success. These procedures can be standardized to reduce issues such as inconsistent spermathecal filling and delayed oviposition in *A. cerana indica.*

The relationships among instrumental insemination, pheromone production, and worker behavior are also unexplored. Queen mandibular pheromone (QMP) profiles differ between virgin and mated queens (Khan et al., 2022), influencing worker behavior and queen acceptance. Given the limited understanding of post-insemination pheromonal development in *A. cerana indica*, research on pheromonal development following instrumental insemination is important.

Technological Innovations for Improving Instrumental Insemination in Honey Bees

Technical constraints associated with manual handling during instrumental insemination may be overcome through technological innovations such as automated insemination system. Microfluidic handling systems could enable precise control of semen volume, pressure, and flow dynamics, thereby reducing operator-dependent variability and minimizing mechanical damage to sperm. Automation of syringe movement, queen positioning, and insemination depth may further improve speed, precision, and consistency in insemination procedures.

Recent anatomical and histological imaging studies in *A. mellifera* further demonstrated that the valve fold structure can obstruct insemination capillaries and influence sperm deposition efficiency, highlighting the importance of refining insemination devices and queen-handling techniques (Dziechciarz et al., 2025). Such findings may contribute to the development of more precise and species-specific insemination systems for honey bees. These technological improvements are particularly relevant for large-scale breeding programs, where consistency and standardization are critical.

Recent studies in *A. mellifera* have also challenged traditional assumptions regarding reinsemination of laying queens. Gąbka and Zajdel (2025) demonstrated that egg-laying queens could be successfully reinseminated without interrupting oviposition or negatively affecting queen survival, opening new possibilities for advanced breeding strategies and genetic management in honey bees.

Semen handling and cryopreservation are important areas for future improvement in instrumental insemination because semen processing and storage conditions directly affect sperm viability and fertilization success (Cobey et al., 2013). Although short-term cold storage above freezing has shown practical value in honey bee breeding programs (Cobey, 2016), long-term cryopreservation of drone semen still remains difficult. Honey bee spermatozoa are highly sensitive to osmotic stress, temperature fluctuations, oxidative damage, and the toxic effects of cryoprotectants during freezing and thawing (Hopkins & Herr, 2010).

Over the past two decades, several studies have attempted to improve cryopreservation techniques for honey bee semen. Taylor et al. (2009) investigated different semen diluents, cryoprotectants, and semen dilution ratios in *A. mellifera* and reported that optimized combinations of antioxidant-based diluents and dimethyl sulfoxide (DMSO) improved post-thaw sperm viability. Later, Wegener and Bienefeld (2012) demonstrated that commonly used cryoprotectants such as DMSO and ethylene glycol may negatively affect both spermatozoa and queens by reducing the number of sperm reaching the spermatheca after insemination. In a subsequent study, Wegener et al. (2014) developed a modified cryopreservation method involving dialysis-based cryoprotectant addition and reduced semen dilution, which enhanced post-thaw fertility and increased the proportion of female brood produced after insemination.

Recent advances in semen preservation research have focused on improving semen extenders and reducing cryogenic damage through antioxidant supplementation. Rajamohan et al. (2020) developed a non-activating semen diluent capable of prolonging in vitro sperm viability and improving semen preservation following cryopreservation. Similarly, Nur et al. (2020) reported that supplementation of freezing media with trehalose improved post-thaw sperm motility and plasma membrane integrity in honey bee drones. Studies on Africanized honey bees further demonstrated that Tris-based extenders were effective in maintaining sperm morphology and membrane integrity after thawing, highlighting the importance of developing suitable species- and strain-specific extenders for successful semen preservation (da Silva Morais et al., 2023).

More recently, Kaya et al. (2025) investigated the effects of different cryoprotectants, including dimethyl sulfoxide (DMSO), glycerol (GLY), dimethylacetamide (DMA), and ethylene glycol (EG), used individually and in combination. Their study showed that combined cryoprotectant treatments, particularly DMSO-based combinations supplemented with polyvinylpyrrolidone (PVP), resulted in better post-thaw sperm motility, plasma membrane integrity, and spermathecal sperm viability than individual cryoprotectants.

Since most cryopreservation studies have been conducted in *A. mellifera*, further research is needed to optimize semen extenders, cryoprotectant concentrations, freezing rates, and thawing protocols specifically for *A. cerana indica*, whose sperm physiology and semen characteristics differ considerably from those of *A. mellifera*. Future studies should therefore focus on developing low-toxicity cryoprotectant systems, improving semen extenders, minimizing oxidative and osmotic damage during freezing and thawing, and enhancing post-thaw sperm migration and storage within the spermatheca. The application of advanced sperm-quality assessment techniques, such as fluorescent viability assays and computer-assisted sperm analysis (CASA), may further improve the standardization and reliability of cryopreservation protocols. Successful long-term preservation of honey bee semen could support semen banking, conservation breeding, preservation of locally adapted populations, and long-term conservation of valuable honey bee germplasm through instrumental insemination.

Integrating molecular breeding tools with instrumental insemination offers significant opportunities for genetic improvement in honey bees. Molecular markers associated with traits such as disease resistance, hygienic behavior, honey production, gentleness, and climate tolerance can be used to guide controlled mating programs and facilitate the transmission of desirable alleles. Instrumental insemination is particularly valuable in this process because it allows complete control over mating and accurate identification of paternal genetic contributions, which is not possible under natural mating conditions. Recent advances in genomic technologies, including high-density single nucleotide polymorphism (SNP) arrays, genome-wide association studies (GWAS), and whole-genome sequencing, have further improved the precision and efficiency of honey bee breeding programs (Jones et al., 2020). The development of a high-density 100K SNP array for honey bees has enabled more accurate genomic selection, quantitative trait analysis, and identification of markers linked to economically important traits such as hygienic behaviour, honey yield, gentleness, and *Varroa* resistance (Jones et al., 2020). Thus, instrumental insemination provides an effective link between genomic information and practical honey bee breeding strategies by enabling controlled transmission of selected genetic traits.

Recent advances in gene-editing technologies may further expand the future applications of instrumental insemination in honey bee improvement programs. CRISPR/Cas9-based genome editing has emerged as a promising tool for studying gene function and potentially improving economically important traits in honey bees and other insects. A recent study by Yıldız and Karabağ (2025) demonstrated successful liposome-mediated CRISPR/Cas9 delivery through drone sperm cells, thereby overcoming some of the technical difficulties associated with embryo microinjection in honey bees. The study also reported successful generation of gene-edited offspring following artificial insemination of queens with transfected sperm, highlighting the future potential of integrating gene-editing technologies with instrumental insemination systems. The integration of molecular breeding, genomic selection, and gene-editing technologies with controlled insemination systems may therefore support the development

of more resilient, disease-tolerant, and climate-adapted honey bee populations in the future.

Developing species-specific training programs, clear technical guidelines, and standardized success metrics will ensure that instrumental insemination produces reliable and consistent results across different users. When the technique is standardized, it becomes easier to learn and use, thereby encouraging more beekeepers and researchers to adopt it. This is especially important for *A. cerana indica* breeding programs in Asia, where instrumental insemination is still rarely practiced and lacks well-established protocols.

In the era of increasing environmental stress and biodiversity loss, instrumental insemination is becoming increasingly important for conservation breeding and genetic resource management in honey bees. Improved protocols could aid in preserving locally adapted *A. cerana indica* populations, while continued refinement of *A. mellifera* instrumental insemination techniques will support global breeding programs aimed at enhancing long-term colony resilience.

Conclusion

Instrumental insemination in *A. mellifera* has reached a high level of standardization, supported by favorable reproductive anatomy and decades of technical refinement. In the case of *A. cerana indica,* the application of this technique remains constrained by species-specific biological limitations, including small body size, low semen yield, heavy mucus contamination, and reduced post-insemination sperm migration. These challenges create both biological and technical barriers that cannot be overcome through direct transfer of protocols developed for *A. mellifera*. The limitations observed in *A. cerana indica* do not arise from a lack of methodology, but rather from insufficient species-specific optimization. Future progress requires redesigning insemination strategies, including low-volume repeated dosing, refined semen handling and dilution protocols, and the development of micro-instrumentation adapted to the reproductive anatomy of this species. The integration of molecular and genomic tools into breeding programs may further accelerate genetic improvement and aid in the conservation of locally adapted populations. Thus, instrumental insemination should be viewed as a biology-dependent breeding tool whose effectiveness is governed by species-level reproductive traits rather than as a universally standardized technique. Addressing these constraints is essential for advancing selective breeding, conserving genetic resources, and sustaining *A. cerana indica* populations, particularly in regions where this species plays a critical ecological and apicultural role. Strengthening breeding programs for this native pollinator ultimately contributes to resilient agricultural systems, biodiversity conservation, and long-term food security.

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Authors' Contributions

A.S.R.: investigation, data curation, writing-original draft, writing-review & editing.
M.R.S.: conceptualization, methodology, writing-review & editing, supervision.
V.R.S.: resources, project administration.
N.M.B.: writing-review & editing, validation, formal analysis
S.M.: validation, formal analysis.
V.D. and D.M.: visualization and formal analysis
A.M.R.K. and M.B.G.: writing-review & editing, validation.

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