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INSTRUMENTAL INSEMINATION AND SEMEN CRYOPRESERVATION: CAN HONEY BEES BE SELECTIVELY BRED LIKE CATTLE?

Mills, S. , Agricultural Extension Agent, MSU

ABSTRACT

Recent advancements in the instrumental insemination of queens, and cryopreservation of honey bee semen have implications that may affect the future of honey bee breeding and the sustainability of honey production. Honey bee breeding is complicated by the haplodiploidy genetic architecture of the colony created from a diploid polyandrous queen and her random mating with several haploid males. Further hampering of breeding programs is generated by the method by which desirable traits are articulated within the colony, whether from genetic heritability, environmental influences, or behavioral effects. Despite these complications, the future of honey bee breeding is at the cusp of amelioration.

INSTRUMENTAL INSEMINATION

Instrumental Insemination (II) of honey bee queens is not new science. The technology was developed in 1927 by Dr. Lloyd Watson and perfected in the 1940's and 1950's (Cobey,1983; Laidlaw, 1987), though until recently, instrumentally inseminated queen survival and productiveness were limited. Infections, insufficient semen placement, poor brood pattern, queen mortality, and hive acceptance were issues that plagued the II program (Cobey, 2007; Cobey, 2009; Cobey, 2016a,b). With improved care of the queens, proper age of insemination, and following strict sanitation protocol, II queens are as successful if not more successful than naturally mated queens (Cobey, 2016a,b). II has been utilized in research for over 50 years, but according to Cobey (2016a,b) the process is underutilized by queen rearing beekeepers. Part of this reticence is due to misconceptions about the performance of artificially inseminated queens. Cobey's work has been aimed at improving the techniques, knowledge and skills of the queen breeder in an effort to improve bee breeding capabilities nation-wide. Cobey has shown that II can be taught to beekeepers interested in developing and working toward improving their breed lines.

Several critical elements of II queens and semen collection are required for II queens to be successful. Based on a review of II studies, Cobey was able to identify those factors as: 1) the quality of the queen; 2) the age of the virgin queen should between 5 and 10 days when inseminated; 3) placing the queen with workers before insemination is important for proper nutrition; 4) insemination with sufficient amounts of semen to signal fecundity to the colony; and 5) placing the queen with workers following insemination is critical for the migration of the sperm from the oviducts into the spermatheca and promotes the onset of oviposition. The amount and timing of carbon dioxide used to anesthetize the queen during the insemination process and care of the semen as discussed below is also of importance. Cobey (2016a,b) has found that these issues can be controlled and improved so II Queen survival and success is maximized.

SEMEN COLLECTION AND CRYOPRESERVATION

New breakthroughs in semen cryopreservation have improved the ability to store, collect, and preserve semen from populations throughout the world. Cryopreserved honey bee semen allows insemination at any time of the year, is easily transported, and reduces the chance of introduction of pests and diseases associated with live bee transport (Paillard *et al*., 2017). Several factors may contribute to poor brood production from cryopreserved semen: 1) size and age of the drone at semen collection (Metz and Tarpy, 2019); 2) quality of the semen (Cobey, 2016a,b); 3) cryoprotectant and extenders used (Taylor *et al.,* 2009; Hopkins and Herr, 2010; Wegner and Bienefeld, 2012); 4) presence of oviduct clogging mucus in the semen (Cobey *et al*., 2013) 5) handling of the semen during collection and insemination (Collins, 2004; Cobey, 2013) and 6) number of drones contributing to the semen (Gerula *et al*., 2014).

Freezing semen in liquid nitrogen for preservation and ease of transport has long been utilized in mammalian breeding programs, but there are problems with cryopreservation of honey bee semen. Cryoprotectants used in liquid nitrogen preservation have proven to be detrimental to queens and/or to sperm viability (Mel'Nichenko and Vavilo, 1976; Harbo, 1977, 1979a,b, 1983; Kaftanoglu and Peng, 1984; Peng *et al*., 1992). The standard for now as developed by Harbo (1983) and confirmed by Hopkins and Herr (2010), is the use of 60% semen, 10% DMSO (dimethyl sulfoxide) and 30% saline solution cooled to just above freezing and frozen at a rate of 3°C/min.

Researchers are currently testing several different types of preservatives, but the research is in its infancy. Dadkhah *et al*. (2016) tested the efficacy of using egg yolk as well as two different concentrations of soy lecithin as preservatives. Their findings suggest that egg yolks are the best preservative and a 2%

concentration of soy lecithin was a close second, but both methods had about 50% of the viability of fresh semen (Dadkhah *et al*., 2016). Metz and Tarpy (2019) investigated the quality of the donor drones in the viability and success of II. Their findings suggest that optimum drone age for semen collection is about 20 days with a substantial decline in spermatozoa count after day 30 (Metz and Tarpy, 2019). The study also showed that drone reproductive physiology is more highly variable than previously thought (Metz and Tarpy, 2019).

Gül *et al*. (2017) attempted to remove the DMSO through centrifugation and replace it with either glucose solution, fresh ram semen plasma, fresh honey bee semen plasma, or extender solution. The use of fresh honey bee semen plasma was statistically similar to freeze-thaw semen without centrifugation at 48% brood production, and the extender was only slightly less effective at 40% (Gül *et al*., 2017). Glucose and fresh ram semen plasma were proven to be ineffective diluents at 3.04% and 0.32% brood production respectively, but all treatments were about 50% of fresh semen (Gül *et al*., 2017). The process of centrifugation may contribute to the loss in viable spermatozoa, but a study by Paillard *et al*. (2017) found that centrifugation to remove the cryoprotectants did not affect queen survival, spermathecal sperm counts or sperm viability. Wegener et al. (2014) found that dialysis of previously frozen semen to remove cryoprotectants resulted in up to 79% female brood production from II queens and increased the amount of semen held in the spermathecae.

Cobey *et al*. (2013) found that strict sanitary protocols during semen collection was necessary to reduce contamination by fecal material that can cause infection in the queen and limit spermathecal migration. Collins (2004) found that the collection of the semen from an ejaculated drone into a buffer rather than a syringe, maintained semen viability and the use of a sodium chloride/antibiotic solution for prophylactic purposes during II slightly improved the viability of the sperm.

Gerula *et al.* (2014) compared queens inseminated with semen of drones from 30 different colonies against queens inseminated with drones from a single colony to discern whether the higher genetic diversity resulted in greater development, productivity, and overwintering ability. The study concluded that there was no significant difference in the functional characteristics between the two colonies (Gerula *et al*., 2014); however, higher genetic diversity may be critical for other factors including worker-queen interactions due to superior queen mandibular pheromone levels as described by Richard *et al*. (2005). More important to successful breeding is the volume of semen used in the insemination as identified by Cobey (2007, 2009, and 2016a,b) and Kocher *et al*. (2010).

GENETICS AND RELATEDNESS IN BEES

The genetic configuration of a colony is made up of many related individuals, none of which are related 100% to each other (Oxley and Oldroyd, 2010). There is a high degree of relatedness within subfamilies (same mother and father) of a colony in that they share 75% of their genes with each other, while members of different subfamilies (same mother but different fathers) only share 25% of their genes with each other (Page and Laidlaw, 1988). This is consistent with the haploid father and a diploid, polyandrous queen mother who mates with between 7 and 20 drones, and selectively fertilizes her eggs. The colony biological organization and genetic architecture is comprised of four levels: the gene, the individual, the patriline, and the colony (Reeve and Keller, 1999). Mutations, gene expression and genetic variability at the gene level all influence the behavior and phenology of the colony (Oxley and Oldroyd, 2010). The individual influences the colony via expression of its behavior, disease resistance, metabolism, pheromone production and egg laying capacity (Oxley and Oldroyd, 2010). The patriline level is influential within colony diversity, response stability, foraging preference and task specialization (Oxley and Oldroyd, 2010). Knowledge of a queen's father is important in understanding the drone's contribution to the colony's performance as well as behavioral and physiological traits (Henderson, 1984). Commercially important traits such as honey production, pollination efficiency and wax production are evaluated at the colony level (Oxley and Oldroyd, 2010). A complete understanding of how each of these four levels interact within each level and upon each other is critical for determining colony performance in subsequent generations (Oxley and Oldroyd, 2010).

HERITABILITY OF DESIRABLE TRAITS

Developing a selective breeding program requires identifying superior traits and ensuring that all the alleles for those traits are passed on to the progeny (Oxley and Oldroyd, 2010). The genetic diversity of the colony created by the haplodiploidy genetic architecture, polyandry of the queens, and panmixia (random mating), coupled with the fact that some traits may be expressed via a suite of genes, or through behavioral responses to external influences, makes development of a selective breeding program highly complex (Burlew, 2018). Further, American honey bee populations have a constrained gene pool due to the Honey Bee Act of 1922 where importation of honey bees was forbidden (Cobey, 2009), though some feel that this is an unwarranted claim (Burlew, 2018).

Commercially important traits such as hygienic behavior, superior honey production, and defensive behaviors are moderately to highly heritable (Oxley and Oldroyd, 2010). Most commercially important traits are not expressed through drones, but they can influence development time (Moritz, 1994), pathogen resistance (Behrens et al., 2007), or color (Laidlaw and el-Banby, 1962). Queen-directed heritable traits include egg laying capacity and rate of pheromone production, while worker-directed expression of traits include hoarding behavior, honey production and responsiveness to pheromones (Gupta *et al*., 2013).

Learning in drones (Chandra *et al*., 2001), defensiveness and stinging behavior (Hunt *et al*., 1998; Hunt *et al*., 1999; Guzmán-Novoa *et al*., 2002; Arechavaleta-Velasco *et al*., 2003; Lobo *et al*., 2003; Arechavaleta-Velasco and Hunt 2004), body size (Hunt *et al*., 1998), age of first foraging and foraging preference (Hunt *et al*., 1995; Page *et al*., 1998; Page *et al*., 2000; Rueppell *et al*., 2004; Rüppell *et al*., 2004; Rueppell, 2009) hygienic behavior, worker sterility, and sex determination are all behaviors that have been identified with multiple alleles acting at a quantitative trait locus (QTL), or candidate gene (Oxley and Oldroyd, 2010).

To gain a specific trait in a population the flow of genes into the breeding lines must be restricted (Burlew, 2018). This can be achieved by using "island biogeography" which involves isolating a breeding population so that no external genetic material influences the selection program (Quammen, 2004; Burlew, 2018). Essentially, the virgin queens must be kept from mating with drones outside the targeted colonies. This inbreeding process will increase the likelihood of the preferred genetic material being retained but will also increase the probability of sustaining unwanted genetic traits (Oxley and Oldroyd, 2010; Burlew, 2018).

The most carefully laid breeding programs may be derailed through a new virgin queen mating with drones naturally and increasing the gene flow within a breeding program (Burley, 2018). An example of this is the varroa mite resistance trait in commercial beekeeping situations, where all the related genes are

recessive (Burlew, 2018). Bringing a varroa mite resistant queen into an area where there is unrestricted breeding with many colonies of bees without this trait, will result in the loss of that characteristic within the colony (Kefuss *et al*., 2016).

BREEDING PROGRAMS

The use of instrumental insemination and cryopreserved semen will help bring desired traits into mainstream honey bee biology when the average queen rearing beekeeper can utilize these tools in their own workshop. Washington State University has received a WSARE (Western Sustainable Agriculture Research and Education) grant to teach these techniques through a series of workshops in the Pacific Northwest to queen rearing beekeepers and Extension professionals. They also plan to develop instruction manuals and DVD's on both II and cryopreservation of semen. A conference is being planned in Pullman, Washington for 2021. With more queen rearing beekeepers working toward a common selective breeding program, and more queens introduced with a specific desired characteristic, unrestricted gene flow from colonies without desired traits will be minimized.

Recent advances in the sequencing of the honey bee genome and the development of genetic tools for gene mapping such as QTL, and gene expression analysis (such as microarrays and RNA sequencing), will have significant impacts on the ability to selectively breed honey bees (Niño and Jasper, 2015). Aphid Lethal Paralysis virus, Big Sioux River virus and Lake Sinai viruses 1 and 2 (Runckel et al., 2011) were identified with high-throughput sequencing of RNA extracted from honey bees in the US showing that genomics could be used to identify novel pathogens that may contribute to declines in honey bees (Grozinger and Robinson, 2015).

The use of genetic markers has become the norm for genetic evaluation and selective breeding programs in mammals such as cattle (VanRaden *et al*., 2009), sheep and hogs (Ostersen *et al*., 2011). However, honey bee population dynamics such as numerous patrilines and colony relatedness, pose a problem for the use of genetic markers in the same multi-step procedure used in other livestock, so scientists have developed a single-step, unified approach to genetic evaluation (Gupta *et al*., 2013). The advantages to this approach are a more accurate estimate of breeding values for ungenotyped animals, selection bias resistance, and a much simpler tool that provides extension to a maternal multi-trait model (Gupta *et al*., 2013).

Genome editing tools such as CRISPR and TALEN could lead to transgenic honey bees with greater resilience to pests and environmental stressors (Grozinger and Robinson, 2015). The manipulation of the gut microbes within the honey bee could allow it to be more efficient at nutrient uptake, or could enhance pesticide detoxification (Grozinger and Robinson, 2015). Internal and external parasites that impact honey bee health can also be manipulated through feeding dsRNA (double strand RNA) to honey bees to target specific genes within the pest to reduce infestation loads (Paldi *et al*., 2010). Paldi *et al*., (2010) showed that the use of dsRNA fed to honey bees could be transferred to the endoparasite Nosema and silence the expression of a specific gene within the parasite to reduce the pathogen load on the honey bee. Feeding dsRNA has also been shown to effectively reduce the targeted gene in Varroa mite and lower mite loads in honey bees (Garbian et al., 2012). Continued testing of these techniques may also help prevent the introduction of sequences of genetic material that may impact the honey bees themselves (Nunes et al., 2013; Grozinger and Robinson, 2015).

FUTURE OF HONEY BEE BREEDING PROGRAMS

What does this mean for the future of selective breeding programs for honey bees? According to Burlew (2018), a single beekeeper can shift the flow of genes within a specific area if they have restricted introductions of queens with desirable traits. However, if there are beekeepers within the area that are introducing queens sans the trait, the gene flow will be diluted and potentially lost (Burlew, 2018). Cobey (2016b) saw that the use of instrumental insemination and cryopreserved semen was part of a long-term solution to sustainability of honey bee populations throughout the world. Honey bee breeders now have more opportunities and greater access to tools that were once only available to mammalian species including genome technology and gene transfer techniques (Oxley and Oldroyd, 2010). The use of geographical and temporal isolation combined with instrumental insemination, gene sequencing, and genetic markers will allow honey bee selective breeding to become economically viable (Oxley and Oldroyd, 2010). Novel and innovative techniques of integrated pest management may allow targeting of pests through feeding of highly explicit dsRNA to honey bee workers (Paldi *et al*., 2010). The future sustainability of honey bee populations is going to be impacted by these recent developments.

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