Honey Bee Stock Improvement

Project No.: **09-POLL4-Cobey**

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Objectives:

1. Enhancement of genetic diversity of domestic commercial stocks through the importation honey bee semen from select lines of European bees.
2. Develop a protocol for the safe and practical international exchange of honey bee germplasm.
3. Provide technology transfer of the skills required for honey bee stock maintenance.

Interpretive Summary:

A healthy beekeeping industry is essential to supply the colonies required for almond pollination. Beekeepers continue to experience high losses resulting from pests, parasites, pathogens, and the phenomena of colony collapse disorder. As colony numbers continue to dwindle, an alarming 30% winter loss is now considered the norm. In addition, the routine use of miticides and prophylactic medications in colonies has reduced selection pressure for natural mechanisms of resistance to pests and diseases in our domestic stocks, further weakening them. Consequently, the narrowing gene pool used to restock colonies nationwide is a concern.

The honey bee, *Apis mellifera*, is not native to the U.S. and was founded upon a limited gene pool. Genetic diversity, the raw tools for selection, enhances colony fitness, survival, and lessens the impact of pests and diseases. To advance honey bee stock improvement, the focus of our continuing project is to increase genetic diversity of our domestic honey bee populations and to assist the California queen producers in stock selection and maintenance programs.

Honey bee semen was imported from populations of *Apis mellifera ligustica* from Italy and *Apis. carnica* from Germany, under USDA-APHIS (Animal Plant Health Inspection Service) permit. The imported semen was inseminated to virgin queens reared from domestic commercial stocks. The progeny (virgin queens) of these crosses were then back-crossed to additional semen importations, as well as crossed with domestic commercial stocks.

The crossing of domestic commercial honey bee lines (insemination of queens) with the imported stocks (semen) is designed to assure the lines are productive and well adapted to U.S. beekeeping conditions and management practices. Queen produced from these crosses has been released to collaborating queen producers in California.

Samples of imported semen tested positive for some common viruses also present in the U.S. Progeny tests of the resultant colonies revealed a greatly reduced incidence of virus and were released. The 2008 and 2009 imports of *A.m. ligustica* and *A.m. carnica* stocks have been released from the WSU quarantine station. The 2010 *A.m. ligustica* semen importation, currently in the WSU quarantine yard has recently been approved for release.

Vertical transmission of viruses is known to occur, passing from drones to semen and queens to eggs. The transmission of viruses is often non-symptomatic, and difficult to detect and exclude. However, the importation of germplasm (eggs and semen) poses less risk compared to live queens and package bees. Considering that little is known about the rate of transmission, we are exploring an egg transfer system that may offer a more practical and efficient means for the international exchange of honey bee stocks.

To better understand the transmission of queens to eggs, we have surveyed commercial bee breeding populations in California and Louisiana, to determine the prevalence of virus in queens and their eggs. Our results suggest the mode of transmission from queens to eggs does not appear to be strong and may vary depend upon the specific virus.

Honey bee eggs are good candidates for stock exchange because this provides a complete genetic package, they are prolifically produced, and the three day egg stage allows time for transport. We developed a method to manipulate eggs to allow for their isolation, pathogen testing, long distance transport, in vitro hatching and subsequent grafting for specific-pathogen-free queen production.

Eggs are manipulated using a pair of micro-forceps modified by the application of micro-bore tubing to the distal pincers. The eggs are easily removed from their beeswax combs and placed in sterile plastic plates for transport. We have successfully hatched the transferred eggs in vitro and in vivo to rear the larvae into queens, as described the previous Almond Board research report. Using these techniques we demonstrate that honey bee eggs can be collected, transported and reared into queens in a reliable and practical manner.

A critical aspect of this project is working closely with cooperating queen producers, members of the California Bee Breeders Association, to assure the value and maintenance of honey bee breeding stocks over the long term. This effort is in collaboration with Marla Spivak's University of Minnesota Tech. Transfer Team and

Steve Sheppard's Washington State University (WSU) Honey Bee Health Program. We also provide practical hands-on training, annually conducting specialized short courses in queen rearing and instrumental insemination at UC Davis Laidlaw Honey Bee Lab (UCD).

Materials and Methods:

1. Increasing genetic diversity of domestic commercial stocks through the importation honey bee semen.

Two generations of honey bee semen of *Apis mellifera carnica* from Germany and three generations of *Apis mellifera ligustica* from Italy have been successfully imported and established under USDA-APHIS permit. The resulting colonies were initially established at Smoot Hill, an ecological reserve of Washington State University, WSU, designated as an USDA approved honey bee quarantine area.

Honey bee semen of *A.m. ligustica* was collected and imported from select stocks from Bari in southern Italy in 2008 and 2009, and from Bolonia in northern Italy in 2010. Honey bee semen of *A.m. carnica* from the German Carnica Association was imported from the Kirchhain Honey Bee Institute in Germany in 2008 and 2009.

European honey bee semen was shipped, or hand carried to WSU. Collaborating queen producers supplied various lines of Italian virgin queens from their commercial stocks for insemination to the Italian imported semen. UCD supplied virgin queens from the New World Carniolan Breeding Program (NWC) for insemination to the Carniolan imported semen.

The progeny of the semen imports of 2008 and 2009 have been backcrossed to create more pure stains of the subspecies. These have also been crossed with resident commercial stocks. We are also creating various crosses to incorporate the imported stocks into domestic commercial lines to enhance genetic diversity is an on-going collaboration with participating California queen producers.

2. Develop a protocol for the safe and practical international exchange of honey bee germplasm.

Importation of honey bee eggs would provide a complete genetic package and is more easily obtained. The collection of semen is restricted to the season production of drones and requires specialized expertise and equipment to collect. Coordinated efforts must be made to assure virgin queens of the proper age are supplied for instrumental insemination upon receipt of the semen. The transport of eggs may also minimize the risk of viral transmission as compared to semen, although further research is needed to determine this. Therefore, we developed new technologies for the transport of honey bee eggs.

To better understand the rate of transmission between queens and their eggs, we surveyed commercial apiaries in the major queen production areas of the country. Honey bee queens, six months, or older, and 50 of each queen's eggs were sampled from 100 colonies. The samples represent nine commercial breeding populations from northern California and Louisiana. The resident queen and 50 embryos were collected

from each colony. Samples were recovered from each colony using a new pair of clean nitrile gloves to obtain each sample, which was then placed in sterile micro-centrifuge tube. Samples were immediately chilled to -80^{o}C on dry ice and stored, and shipped at -80^{o}C to the USDA Beltsville laboratory for RT-PCR (reverse transcription-ploymerase chain reaction) analysis of eight known honey bee viruses.

One hundred colonies were sampled for this study. Seventy colonies were sampled in March 2010 from seven northern California commercial queen production operations, ten colonies per business. The California colonies represented various breeding populations including; Italian, Cordovan, Carniolan, Minnesota hygienic and Australian Italian. Thirty colonies were sampled from two breeding sites in Louisiana in April of 2010, twenty colonies of VSH (*Varroa* sensitive hygiene) genetic background and ten colonies of Russian genetic background from apiaries associated with the USDA lab in Baton Rouge, Louisiana.

Each sample of queens and eggs were individually analyzed using RT-PCR by the USDA Lab in Beltsville. Samples were tested for the presence of eight viruses including: acute bee paralysis virus (ABPV); black queen cell virus (BQCV); chronic bee paralysis virus (CBPV); deformed wing virus (DWV); Israeli acute paralysis virus (IAPV); Kashmir bee virus (KBV); sacbrood bee virus (SBV); and slow paralysis Virus (SPV).

3. Provide technology transfer of the skill required for honey bee stock maintenance.

Annually, we are continuing to offer three specialized beekeeping short courses in the skills required to develop and maintain honey bee stocks. Classes are conducted at UCD, Laidlaw Honey Bee Biology Facility and structured to provide hands-on practical field and classroom training. A working model of the Closed Population Breeding Program is used for demonstration. The classes incorporate new technologies into the curriculum as developments are realized.

The three courses offered are:

1. The Art of Queen Rearing, which includes a tour of several commercial queen producers in northern CA.
2. Instrument Insemination & Bee Breeding.
3. Advanced Techniques In Instrument Insemination.

Results and Discussion:

To enhance the gene pool of US honey bee breeding stocks and to provide access to international bee breeding programs selecting for increased levels of resistance to pests and disease, there is a need to develop a standardized protocol for the safe and practical exchange of genetic stocks. Currently, semen is routinely sealed and stored in glass capillary tubes and transported in slotted wood protectors as shown in **Figure 1**. Semen can be shipped anywhere in the world using this method.

We successfully imported semen from breeding programs in Europe under USDA-APHIS permit. The imported semen was inseminated to virgin queens of domestic

honey bee breeding stocks. Our current focus is to work with and augment the recognized honey bee subspecies currently existing in the US.

Honey bee semen of *Apis mellifera carnica* from Germany was imported in 2008 and 2009 and incorporated into the New World Carniolan breeding program maintained at UCD and distributed to queen producers. Semen of *Apis mellifera ligustica* from Italy was imported in 2008, 2009, and 2010 and inseminated to several commercial stocks of Italian bees supplied by cooperating California queen producers. The progeny of these various crosses were then backcrossed to additional importations to create more pure stocks of the two subspecies. The progeny was also out-crossed to domestic commercial stocks to enhance genetic diversity and evaluate their compatibility and performance.

We also attempted to import *A. m. caucasica*. Although genetic traces can be found in the U.S., it is no longer recognizable as a subspecies. We plan to re-establish Caucasian bees in the U.S., as this subspecies is known for its winter hardiness, gentle temperament, and collection of propolis. Propolis contains antimicrobial properties, providing a natural defense against pathogens. Originally, we hoped to obtain this stock from Turkey. Although the USDA-APHIS import permit has been approved, the delay is due to an arduous export process in Turkey. For this reason, we plan to obtain semen from this subspecies, *A. m. caucasica,* across the boarder from Turkey in the country of Georgia in August 2010.

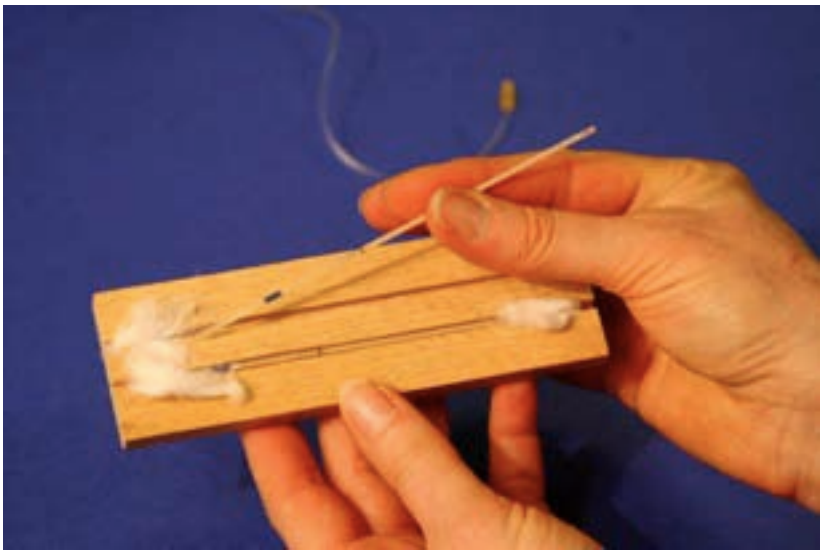

Figure 1: Honeybee semen stored in capillary tubes for transport.

The current semen importation procedure requires that the resultant colonies be established in quarantine for observation and testing. The inseminations were preformed at WSU and colonies established at the Smoot Hill quarantine apiary. The quarantine area presents a difficult environment to maintain colonies due to the lack of forage and harsh cold winters. The delay in release of colonies increases the risk of

loosing stock. The winter conditions were problematic for the Italian stock and some colonies of the initial importation were lost. Therefore, the establishment of a more efficient importation protocol for germplasm could allow for the direct release of stock. An egg transfer system may offer a more practical and efficient means for the international exchange of honey bees. While limiting importation to germplasm minimizes the risk of introducing pests and parasites, the transmission of viruses remains a question. To assist government authorities in the regulation and certification of a new import pathway of honey bee germplasm requires assessing the pathogen status of donor colonies. This requires a better understanding bee physiology and pathology and colony health. To better understand the pathogen relationship, relating the pathogen status of breeder queens and their eggs, we surveyed commercial colonies in the US.

Our preliminary sampling indicates that transmission of virus varies among hive constituents within a single colony judged to be healthy and without evidence of disease, other than *Varroa*. For this reason, we hoped to determine the level of viral transmission between queens, eggs, drones and their semen and feces using the BEE CHIP, a bee pathogen microarray chip, for detection of pathogens being developed by Dr. J.DeRisi's Lab. at UC San Francisco.

Although the Bee Chip works for adult bees, this technique was not sensitive enough for some of the hive constituents. The Bee Chip involves a hybridization technique detection that is not only based on whether a pathogen is present, but is also depends on the relative amount of pathogen and host material isolated from each sample. Obtaining enough RNA from samples of embryo, feces, and semen proved difficult and the results are unreliable.

In control experiments that were known to be highly virus positive, if the starting material was reduced to 100 ng or below then the virus in samples was no longer detectable in samples known to contain at least four honey bee viruses. For these reasons we repeated the sampling in 2010 and used RT-PCR analysis.

Commercial stocks were sampled to determine the presence and transmission of viruses between queens and eggs. During the spring season of 2010, we sampled 100 colonies, taking the queen and 50 of her eggs from each colony. The samples represent nine commercial breeding populations from the major queen production areas of California and Louisiana.

Our results, as seen in **Table 1**, indicate that of the eight viruses tested; only three viruses, BQCV, DWV and IAPV, were detected in both queens and eggs. Although we do know the viral status of the colonies, five viruses; ABPV, CBPV, KBV, SBV, and SPV were not detected in either queens or eggs. This may indicate that the mode of transmission from various constituents of the colony to the queens is reduced, although further testing is needed to clarify this aspect.

The percentage of detectable virus was lower in the eggs as compared to the queens. DWV was the most prevalent virus, found in 95% of the queens and 56% of the eggs. BQCV was detected in 88% of queens and only 3 % of the eggs. To confirm these results, the assay to detect the rate of BQCV in eggs was repeated and did yield the same results. IAPV, although less prevalent by comparison to DWV and BQCV, appeared to have a higher rate of transmission, detected in 27% of queens and 23% of their eggs. Overall, these preliminary results suggest the mode of transmission of virus from the queen to her eggs does not appear to be strong and may vary among viruses.

Table 1: Transmission of viruses between queens and their eggs determined by RT-PCR analysis. The percentage of detectable viruses found in the queen and 50 of her eggs from 100 colonies representing breeding populations in California and Louisiana.

	ABPV	BQCV	CBPV	DWV	IAPV	KBV	SBV	SPV
Queens	0%	88%	0%	95%	27%	0%	0%	0%
Eggs	0%	3%	0%	56%	23%	0%	0%	0%

To allow for the more practical use of egg transfer in bee breeding programs we refined the procedure to allow for the culture of isolated eggs. The transfer of eggs from beeswax comb has been difficult and marginally successful because of their delicate nature and the glued basal end to the comb. We developed a technique to manipulate eggs using a pair of micro-forceps modified by the application of flexible micro-bore tubing to the distal pincers. The apical end of the egg is gently grasped between the tubing and lifted to separate the glued basal end of the egg from the brood comb, pictured in **Figure 2**. Using this technique eggs are easily transferred to sterile plastic plates and maintain their upright position as depicted in **Figure 3**.

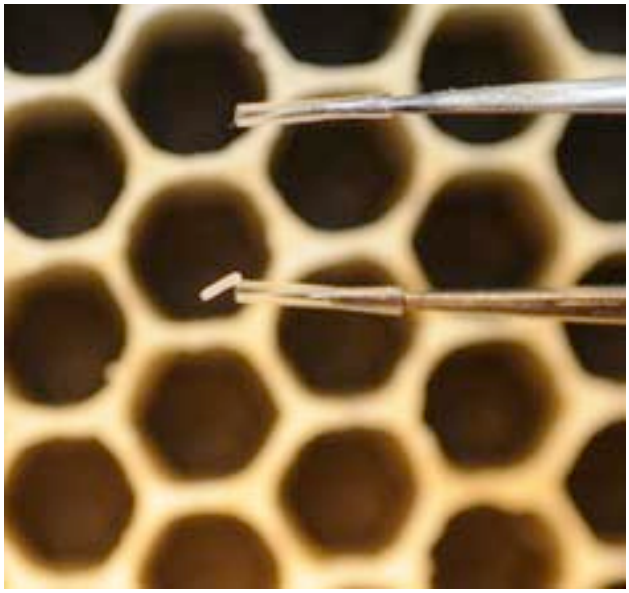

Figure 2: Transfer of an egg using micro-forceps with the attached flexible micro-bore tubing to the distal pincers.

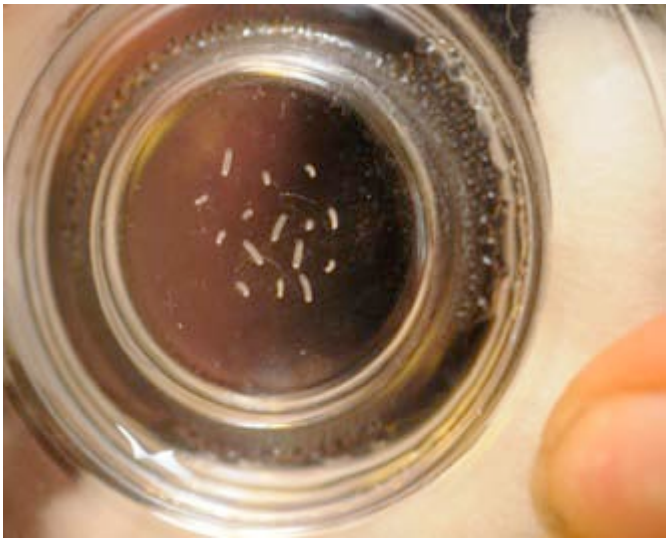

Figure 3: Honey bee eggs placed in a sterile plastic plate.

To allow for greater practical use in commercial bee breeding programs, we refined methods to culture isolated eggs within cell builder colonies once they would arrive at the sight of import. In our previous study, larvae were hatched in vitro, grafted into queen cell cups within 2 hours of hatch and were placed in cell building nursery colonies for queen rearing. The difficult step of grafting small, delicate larvae 2 hr. post-hatch has been eliminated. To improve the technique, isolated eggs transferred into well plates were pressed into the comb of cell builder colonies and hatched in the colonies. This improved the acceptance rate of 51% for in vitro hatched eggs to 85% for in vivo hatched larvae when grafted into queen cell cups for queen rearing.

To allow for transport time, we also cultured the eggs at room temperature in a humidified envelope for a 12-hour period and then transferred the newly hatched larvae to a cell builder colony. Groups of eggs were also cultured in plates and incubated on the surface of a human body with a neoprene wrap for a 12-hour period and then transferred to a cell builder colony (**Figure 4**). The neoprene body wrap would allow for the easy transport of eggs without the need to carry an incubator.

Figure 4: Honey bee eggs incubated in a plate on the surface of a human body with a neoprene wrap.

In the nursery cell builder colonies bees removed the majority of transferred eggs in individual well plates within 2 hours of transfer. Additional experiments subsequently demonstrated that the placement of multiple embryos per well overcame the trigger for egg removal when a minimum of four eggs per well were present. Results (**Table 2**) demonstrated that eggs can be isolated, transferred to micro-well plates, cultured either at room temperature or at body surface temperature (35^{o}C) and then transferred to a cell builder colony to be reared into queens. An acceptable egg hatch rate of >75% and an acceptance rate above 80% for grafted larvae were obtained in these trials.

ET System	Eggs Cultured	Recovered %	Hatched%	Larvae Grafted	Larvae Accepted%
ET In Vitro	2000	2000(100%	1840 (92%)	712	348 (49%)
ET-In Vivo	2340	1889 (82%)	1394 (74%)	728	633 (87%)

Table 2 Production of queens derived from in vitro and in vivo hatched larvae.

Research Effort Publications & Presentations:

Results of this project are currently being prepared for publication. Proceedings and Presentations include:

Susan Cobey. Cooperative stock maintenance and development of protocol for international exchange of honey bee germplasm. Apimondia International Beekeeping Congress, Montpellier, France, Sept. 2009.

John Pollard. Development of embryo transfer technologies in the honey bee for specific pathogen-free queen production and international genetic movement. Apimondia International Beekeeping Congress, Montpellier, France, Sept. 2009.

Susan Cobey. Developing a Protocol for the International Exchange of Honey Bee Germplasm.
California State Beekeepers Assoc. Nov. 2009
American Honey Producers Assoc. Jan. 2010
American Assoc. of Professional Apiculturists. Jan. 2010
Susan Cobey. Closed Population Breeding & Honey Bee Genetic Diversity. Buckfast Bee Breeders Annual Meeting. Germany, March 2010

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