Honey Bee Stock Improvement Program

Project No.: **08-POLL4-Cobey**

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Objectives:

1. To enhance genetic diversity of domestic honey bee stocks through importation and selection programs to increase the level of resistance to pests and diseases.
2. To develop commercially viable reproductive technologies and protocol for the safe international exchange of honey bee germplasm.
3. To provide training in techniques to advance the development, establishment and maintenance of productive commercial breeding stocks with economically valued traits.

Interpretive Summary:

The goal of this project is to: increase genetic diversity of honey bee stocks in the United States; to find safe, practical and more effective means of ensuring imported stocks do not introduce new pests or disease; and to provide beekeepers with skills in stock improvement and the knowledge necessary to incorporate these techniques into their business operations.

Enhancing genetic diversity and selection for traits of resistance to pests and diseases holds a promising long-term solution to maintaining the heath of the honey bee industry. To augment commercial stocks we imported, under USDA-APHIS (Animal Plant Health

Inspection Service) permit, honey bee semen of *A.m. ligustica* from Italy and *A.m. carnica* from Germany. This was mated to virgin queens reared from proven commercial stocks. The German stock crossed with New World Carniolan expressed a high degree of hygienic behavior and is productive and gentle. The Italian stock is currently undergoing evaluation.

Finding safe and effective means of introducing selected honey bee stocks from sources outside of the United States is imperative to minimize or eliminate the potential risk of introducing new pests and pathogens. Honey bee gametes (eggs and semen) eliminate the threat of parasites and many diseases; however, they are known to carry viruses.

Importation of live honey bees (queens and package bees) presents the greatest risk for introducing honey bee pests and pathogens. Importation of semen is currently the safest means of introducing honey bee stock. However, the screening process for testing for viruses requires queens inseminated with imported semen be placed in quarantine while semen samples are tested for known viruses. The quarantine areas present a difficult environment to maintain colonies, delay the release and evaluation, and increase the risk of losing valuable stock.

Considering the high rate of queen egg production, 1000 to 2000 per day, and that the embryo provides a complete genetic package, our focus was to develop practical methodology to transport eggs. Manipulation of honey bee embryos would allow for their isolation, pathogen testing, long distance transport, *in vitro* hatching and subsequent grafting for specific-pathogen-free queen production. Importation of eggs may also prove to be a more effective means of stock transfer if viruses are found not to be incorporated into the cytoplasm of the egg and limited to the outside chorion “shell” of the egg where they can be washed before transfer for queen rearing. Similar techniques are successfully and routinely used with (cows, pigs, goats etc).

Materials and Methods:

1. Enhancement of Commercial Stocks

Permits were obtained from USDA-APHIS to import honey bee semen for a three year period, 2008-2010, from three honey bee subspecies, *Apis mellifera ligustica* from Italy, *A. m. carnica* from Germany and *A. m. caucasica* from Turkey. In consideration of the impact of introduced pests and pathogens and the recognized need to minimize risks, our request was limited to honey bee semen. Honey bee viruses are known to occur in semen; therefore USDA-approved quarantine areas were established to isolate the imported stocks. Smoot Hill, an ecological study reserve of Washington State University (WSU) was established as a USDA approved quarantine area.

In cooperation with Dr. Steve Sheppard, honey bee semen of *A.m. ligustica* was collected and imported from several selected stocks in Italy in 2008 and 2009, and shipped to WSU. Collaborating commercial queen producers supplied virgin queens, which were inseminated with the imported semen in 2008. Honey bee semen of *A.m.*

carnica from the German Carnica Association was imported in 2008. The semen was inseminated to virgin queens from the New World Carniolan Breeding Program (NWC) maintained at UC Davis. The inseminated queens were established in colonies at the Smoot Hill quarantine yard. Daughters of the 2008 queens were reared and inseminated to the semen imported in 2009 and the process repeated. Plans are to repeat this again in 2010.

The virgin queens supplied for this project were from producers involved in a cooperative program between California queen producers and Dr. Marla Spivak from the University of Minnesota, who in collaboration with the UC Davis team are working to identify and encourage the selection of commercial stocks with traits of resistance to pests and disease. Stock from UC Davis was from the NWC program, a closed population of honey bees selected for over twenty-five years.

We also planned to import semen of *A.m. caucasica* from Turkey. Traces of this particular subspecies are detectable in the US gene pool, but have been largely lost. Cobey traveled to Turkey in August 2008 (invited and sponsored by the TEMA foundation ((http://www.tema.org.tr/)) and observed several ecotypes of this subspecies. Although we held USDA permits, we were unable to obtain permits to export semen from Turkey and are waiting pending applications. Valuable contacts and future plans are being developed.

To determine if viruses were present in the imported semen, samples of the semen from Italy and Germany were sent to the USDA Beltsville Honey Bee Lab. for testing. The 2008 Italian stock tested positive for viruses and remained in quarantine throughout the winter. Progeny testing resulted in the release of 3 colonies, which were moved to California for further evaluation. The 2008 German *carnica* stock was approved by APHIS for release. This stock is currently being evaluated and propagated at UC Davis. A limited release was made to participating California queen producers. The 2009 imports of *A.m. ligustica* and *A.m. carnica* stocks have been established and are currently in the Washington quarantine yards undergoing testing.

2. Reproductive Technology Development

Honey bee embryos are good candidates for transfer due to their high rate of production and 3 day stage. Hatched larvae are difficult to manage and die a few hours outside the colony. We focused on developing methodology to manipulate embryos in which we could transfer these in isolation, test them for pathogens, successfully transport over long distances, and then hatch them *in vitro* and rear the larvae into specific-pathogen-free queens.

The transfer of honey bee eggs, up to this point, has been difficult and marginally successful because of their delicate nature. Collins (2003) achieved a 45% survival to hatching *in vitro* rate utilizing Tabor's modified forceps (1960) technique of removing the egg still attached to the beeswax base of the cell. We explored methods to improve the success rate and to allow transfer of eggs to a sterile plastic dish, to eliminate the risk of transfer of pathogens from the wax comb.

Honey bee gametes are known to carry viruses, which still presents questions of risk management. To determine the level of health risk in transporting semen and eggs we are looking at the virus profile of various aspects of field colonies using molecular techniques. In collaboration with Dr. Michelle Flennike, we plan to develop a screening process for the presence of specific pathogenic bee viruses and investigate pathogen elimination procedures for bee gametes.

To test the pathogen relationships within individual colonies, 20 colonies in four different apiaries were sampled as diagramed in **Figure 1**. From each colony the samples collected included the queen, queen wing tips, 10 embryos, 3 larvae, 3 Varroa mites, 3 drone pupae, 3 worker pupae, and semen and feces from 3 drones. Samples are currently being analyzed for honey bee pathogens using the Bee Pathogen Chip developed by Dr. J. DeRisi's Lab. at University of California San Francisco (Wang et al, 2003).

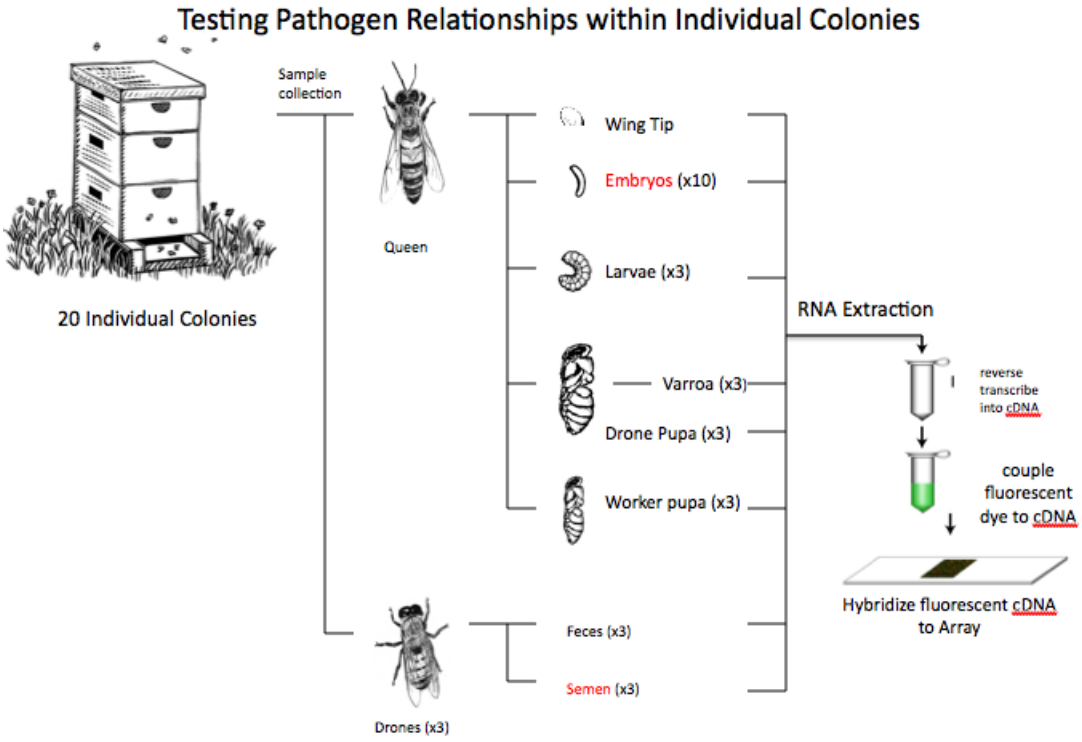


Figure 1. Testing pathogen relationships within individual colonies.

3. Technology Transfer

Three specialized beekeeping short courses were developed and designed to provide the beekeeping community with the skills required to develop and maintain honey bee stocks. These were offered in the spring of 2008 and 2009 at UC Davis, Laidlaw Honey Bee Biology Facility. Courses were structured to provide practical field and classroom training. The working model NWC breeding program was used for demonstration purposes.

The three courses offered:

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

Results and Discussion:

1. Enhancement of Commercial Stocks

Honey bee semen of *A.m. ligustica* from Italy and *A.m. carnica* from Germany was imported and mated to virgin queens reared from proven commercial stocks supplied by cooperating California queen producers. The 2008 NWC X German *carnica* queens were released from the USDA approved quarantine location in Washington and established at UC Davis during the summer of 2008.

Preliminary results suggest these crosses are productive and gentle. Expression of hybrid vigor resulted in very solid brood patterns. To determine the level of hygienic behavior, a mechanism of resistance to *Varroa destructor* and brood diseases, a freeze killed brood assay was used. Removal of dead brood is correlated to removal of diseased and infested brood. A high degree of expression of this trait was observed. Both stocks, NWC and German *carnica* were selected for this trait. The rate of removal of the freeze killed brood, partial and full removal of 160 cells at 24 hours and 48 hours is presented in **Table 1**.

Hygiene	Date	16-Sep-08	Operation						CELLS IN CIRCLE	160	
			START	24 HR CHECK (date:				48 HR CHECK (date:			
Queen	Mother	semen	unsealed	# Sealed	# Partial	%clean+part	% cleaned	# Sealed	# Partial	%clean+part	% cleaned
B31	G62/B4	6/7-45-176-05	20	33	22	76%	61%	13	5	91%	87%
B32	Y51/Y47	6/7-45-176-05	59	23	0	77%	77%	7	0	93%	93%
B33	G68/B88	6/7-45-176-05									
B34	G82/R34	6/7-45-176-05									
B35	G76/W4	6/7-45-176-05	45	0	0	100%	100%	0	0	100%	100%
B36	G76/W4	6/7-146-21-05	64	2	10	98%	88%	0	0	100%	100%
B37	G76/W4	6/7-146-21-05	59	0	0	100%	100%	0	0	100%	100%
B38	G78/R35	6/7-146-21-05	17	6	25	96%	78%	0	0	100%	100%
B39	G76/W4	6/7-146-21-05	4	0	1	100%	99%	0	0	100%	100%
B40	G68/B88	6/7-146-21-05	21	49	30	65%	43%	24	12	83%	74%
B41	G62/B4	6/7-146-21-05	54	0	0	100%	100%	0	0	100%	100%
B42	G68/B88	6/7-146-21-05	19	35	2	75%	74%	1	0	99%	99%
B43-SS	Y83VSH	6/7-146-21-05									
B44	G68/B88	6/7-146-21-05	46	7	2	94%	92%	0	0	100%	100%
B45	Y51/Y47	6/7-146-21-05	38	17	10	86%	78%	1	3	99%	97%
B46	G78/R35	6/7-146-21-05	23	18	16	87%	75%	0	0	100%	100%
B47	Y2VSH	6/7-146-21-05	29	4	6	97%	92%	0	0	100%	100%
B48	G82/R34	6/7-45-176-05	21	4	2	97%	96%	0	0	100%	100%
B49	G62/B4	6/7-45-176-05	41	11	3	91%	88%	1	0	99%	99%
B50	Y51/Y47	6/7-45-176-05	27	20	1	85%	84%	10	0	92%	92%
B51	G82/R34	6/7-45-176-05	30	0	4	100%	97%	0	0	100%	100%

Table 1. Rates of Removal of Freeze Killed Brood at 24h and 48h by Colonies Headed by NWC X German *carnica* Queens.

Colonies headed by queens inseminated to the 2008 imported Italian semen were over-wintered at the Washington quarantine location and progeny was screened for viruses

in the spring of 2009. Many colonies did not survive the severe winter for which this stock is not well adapted. Also the queens were given small semen doses resulting in some becoming drone layers or being prematurely superseded. Of the surviving colonies released from quarantine in spring of 2009, three were moved to California. Daughter virgin queens were propagated from these and mated to the semen imported from Italy in 2009. These are currently in quarantine.

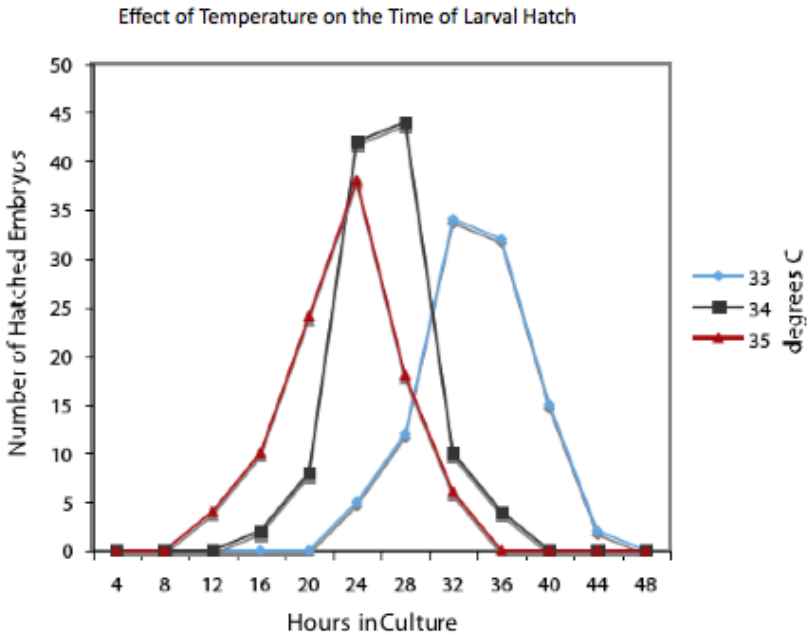
2. Reproductive Technology Development

We developed a new method for the manipulation of embryos improving the rate of success. Micro-forceps were modified by the application of flexible micro-bore tubing to the distal pincers. The extreme apical end of individual embryos were gently grasped between opposing segments of the flexible tubing and then lifted to separate their glued basal end from the comb. The embryo transfer forceps are pictured in **Figure 2**. Using this technique eggs can be easily transferred in isolation to plastic culture dishes. The embryos maintain their upright position, and re-glue to the dishes. This eliminates the need maintain the attached wax base which may carry pathogens.

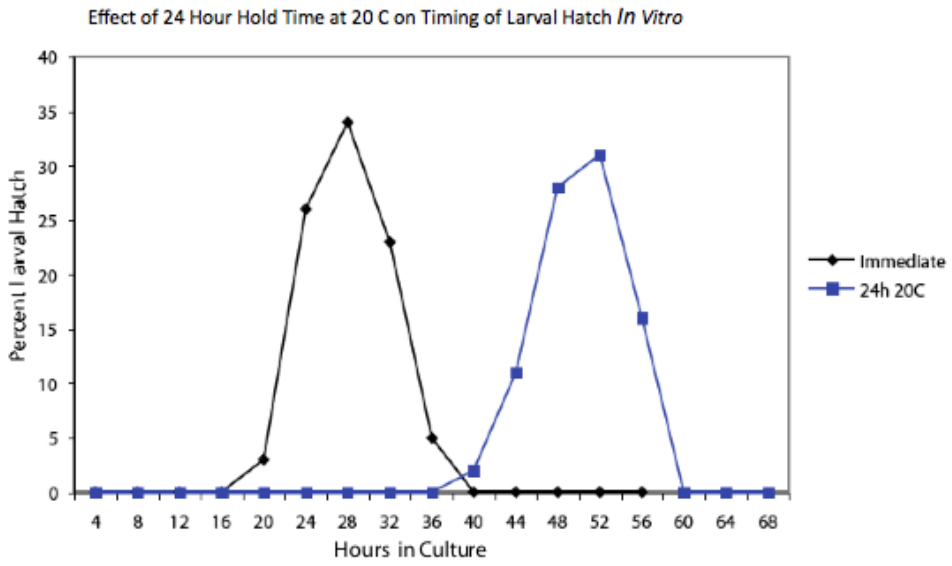
Figure 2. Embryo Transfer Forceps with Mircobore Tubing and Egg.

To understand the limits of this technique for long distance transport we explored the effects of temperature on egg hatch. The honey bee embryo stage is 72 hours. Embryos 50-54 hours post-oviposition are cooling tolerant and were obtained at this stage by confining the queen to a cage with comb and access to worker bees for a timed period. Embryos from individual donor queens (n=10) were transferred in groups (n=100) to sterile plastic culture dishes. The embryos were naturally re-glued in a standing position on the bottom surface of the plastic dishes and placed into 35^{o}C incubators with 95% humidity, air atmosphere for 32 hours. Selected *in vitro*-hatched larva, demonstrating spiracular movement, were grafted into queen cell cups and transferred to cell builder colonies to rear the larvae to queens.

The temperature effects on the time of larval hatch were assessed to determine the limits and timing of egg incubation. A one degree change would slow or speed larval hatch by several hours, as presented in **Graph 1**. Embryos held at 20º C for 24 hours shutdown, but would recover and hatch with a temperature increase, as presented in **Graph 2**.



Graph 1. Effect of Temperature on the Time of Larval Hatch.



Graph 2. Effect of 24 Hour Hold Time at 20º C on Timing of Larval Hatch *In Vitro*

Grafted larvae were assessed for acceptance at 48 hours and 120 hours post-grafting into queen cells and for queen emergence following transfer to emergence cages. Results of trials in 2008, of 998 embryos cultured, and in 2009, of 1734 embryos cultured demonstrated that 94% and 92% of transferred embryos hatched *in vitro* within 32 hours of culture. Of the larvae grafted for queen production, 42% and 40% were accepted and emerged as morphologically normal queens. These queens, 107 in 2008 and 137 in 2009 were instrumentally inseminated and introduced into colonies with normal acceptance rates of 86% and 78% respectively. The mean rate of initiation of oviposition was 5.5 and 6.0 days, as presented in **Table 2**.

Viability Testing of Transferred Embryos

Results of the *In Vitro Culture* of Transferred Embryos and Grafting of *In Vitro* Hatched Larvae

Year	Total Embryos Cultured	Hatched (%)	Total Larvae Grafted	Larvae Accepted	Queens II'd	Accepted	Day of Oviposition
2008	998	941 (94%)	570	239 (42%)	107	92 (86%)	5.5
2009	1734	1595 (92%)	639	250 (40%)	137	106 (78%)	6

Table 2. Viability Testing of Transferred Embryos

To manage the safe movement of honey bee embryos a system was developed to provide a more practical and successful method of transfer, which allows the isolation and *in vitro* hatching of eggs and their subsequent grafting and production into viable queens. The proposed Embryo Transfer System (ET-1) is outlined in **Figure 3**.

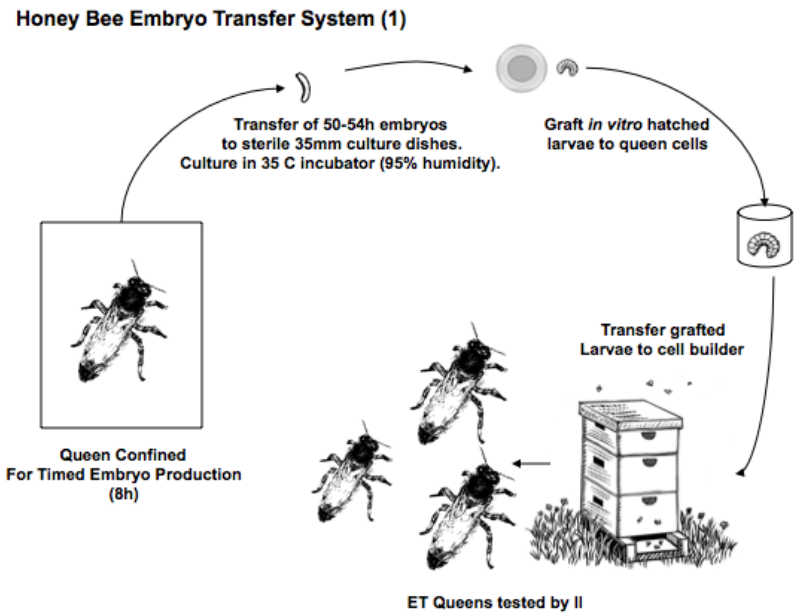


Figure 3. Honey Bee Embryo Transfer System 1

The queen is confined to obtain known age eggs. The eggs are transferred at 50 to 54 hours into sterile 96 well plates maintained at 20ºC for 24 hours. Within two hours of egg hatch in the incubator, larvae are grafted into plastic queen cells and introduced into cell builder colonies for queen rearing. A mean acceptance rate of 51% was obtained for *in vitro* hatched larvae, which did not differ significantly from control larvae. The low rate of acceptance is due to the lack of feed and sensitivity to drying of 2 hour old larvae. The queens reared from *in vitro* hatched larvae appeared morphologically normal and were subsequently instrumentally inseminated. These queens were established in colonies and functioned normally in terms of initiation of egg laying and brood production.

To improve the acceptance rate of *in vitro* hatched larvae by cell builder colonies and their overall efficacy in the production of viable breeder queens we developed a second embryo transfer system (ET-2) diagramed in **Figure 4**. Embryos were transferred into sterile 96 well plates and pressed directly into the comb of queenless and broodless cell builder colonies where the hatching eggs are immediately attended by nurse bees. This eliminates the step of grafting small, delicate larvae 2 hours post-hatch. The acceptance rate of embryos to hatched larvae increased to 85%. The two systems are compared in **Figure 5**.

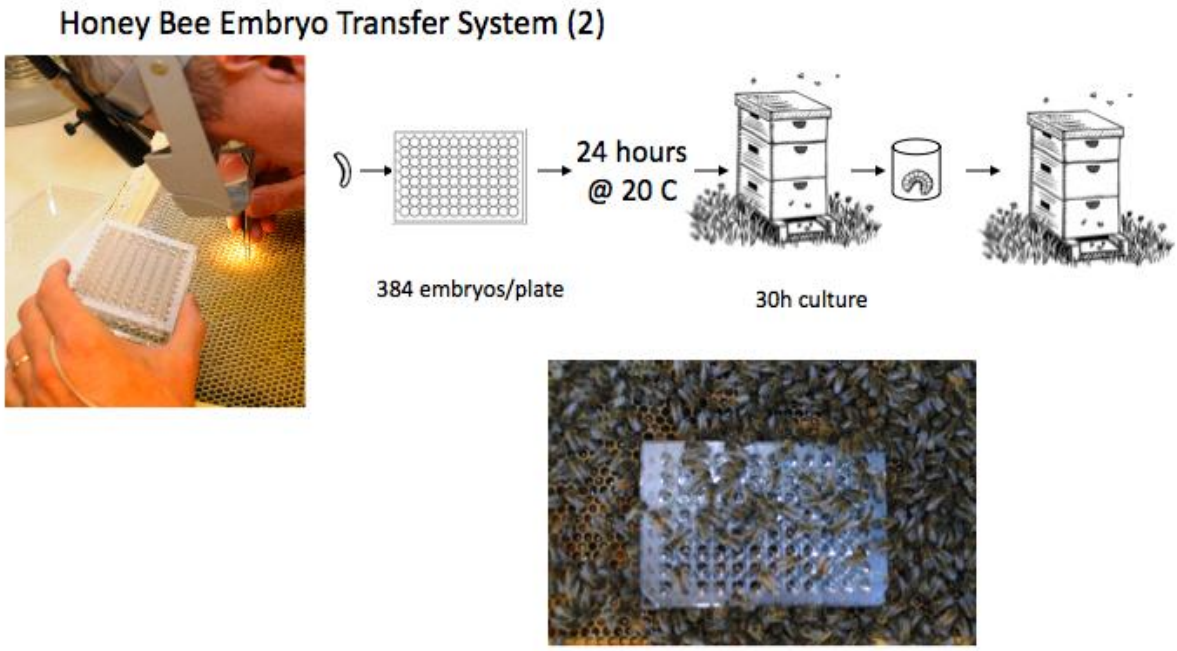


Figure 4. Honey Bee Embryo Transfer System 2. Well plates and pressed directly into the comb of queenless and broodless cell builder colonies.

ET system 1

Year	Total Embryos Cultured	Recovered (%)	Hatched (%)	Larvae Grafted	Larvae Accepted (%)
2008	600	600 (100%)	546 (91%)	270	138 (51%)

ET system 2

Year	Total Embryos Cultured	Recovered (%)	Hatched (%)	Larvae Grafted	Larvae Accepted (%)
2009	2304	1889 (82%)	1394 (74%)	270	229 (85%)

Figure 5. Comparison of *In Vivo* Culture of Transferred Embryos and Acceptance of Hatched Larvae using ET system 1 and ET system 2.

The development of an international embryo transfer program would involve several steps; the initial selection of donor colonies based on genetic superiority and health, molecular pathogen testing, and government certification and approval. Semen would be collected from screened colonies and approved several days prior to queen confinement for embryo production. Embryos would be transferred to well plates and transported over a 24 h period. The plate would then be placed in a cell builder during egg hatch. The larvae would then be grafted into queen cups for queen rearing. The virgin queens would be instrumentally inseminated with the imported stored semen to provide a complete genetic package. The process, diagramed in **Figure 6** would require about one month to complete.

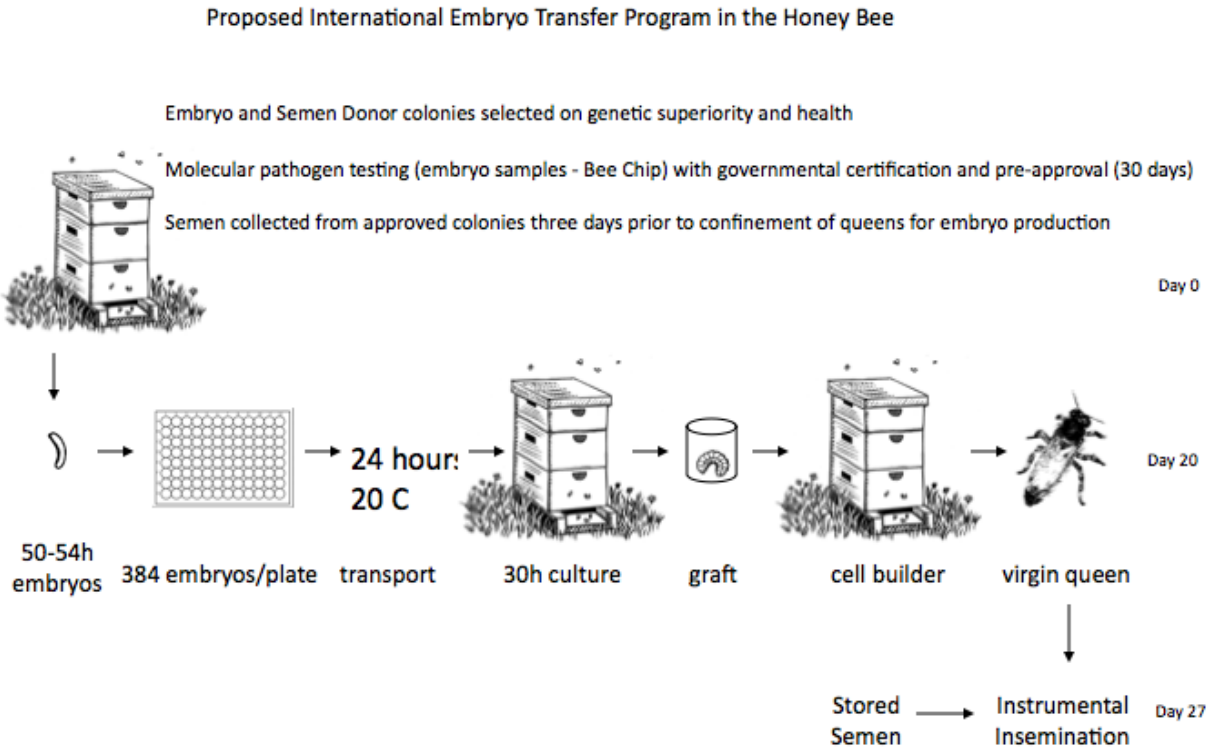


Figure 6. Proposed International Embryo Transfer Program in the Honey Bee

The transmission of virus in honey bee semen and embryos remains a health risk question in management for international transport. Methods for direct pathogen testing of donor colonies and gamete samples would allow for the immediate use of queens produced from imported eggs and semen. In collaboration with Dr. Michelle Flenniken, we are testing a screening process using the Bee Pathogen Chip. Studying the virus profile of various aspects of colonies may provide insight into procedures to eliminate pathogens of bee gametes. We sampled various components, life stages, and castes of 20 individual colonies in four different commercial apiaries, which are currently being analyzed. This project is on-going with future plans to further develop and test

importation protocols to develop safe and effective methods of international exchange of honey bee genetics.

3. Technology Transfer

The specialized beekeeping short courses offered in 2008 and 2009 were well attended with overwhelming response. Beekeepers from across the country and abroad participated. Due to the high demand, a second queen rearing class and third insemination was scheduled in 2009. The signup for 2010 classes are nearly booked. Plans are being made to expand these to meet the demand.

We plan to offer these classes annually and incorporate new technologies into the curriculum as new developments are realized to enhance honey bee stock improvement.

Publications:

Results of this project are currently being prepared for submission to a peer reviewed journal. Results will also be summarized for submission to a beekeeping trade journal.

Abstracts accepted for presentation at Apimondia International Beekeeping Congress,Montpellier, France, Sept. 2009.

Cooperative stock maintenance and development of protocol for the international exchange of honey bee germplasm.
Susan Cobey, John Pollard, Claire Plante, Michelle Flenniken, Walter Sheppard

Development of embryo transfer technologies in the honey bee for specific pathogen-free queen production and international genetic movement.
John Pollard, Claire Plante, Susan Cobey

Development of a prophylactic antibiotic mixture for use with extended honey bee semen during international genetic exchange.
John Pollard, Claire Plante, Susan Cobey

References and Related Citations:

Collins, AM. 2002. Collection of honey bee eggs for cryopreservation. *J. Apic. Res..*41(3-4):89-95

Fuchs, S., & Schade, V. 1994. Lower performance in honey bee colonies of uniform paternity. *Apidologie, 24*, 155-168.

Jones, C.J., Myerscough, M., Graham, S., Oldroyd, B.P. 2004. Honey bee nest thermoregulation; Diversity Promotes Stability. *Science* 305, 402-404

Mattila, H. R., & Seeley, T. D. 2007. Genetic Diversity in Honey Bee Colonies Enhances Productivity and Fitness. *Science,* 317, 362-364.

Schiff, NM., & Sheppard, W. S. 1995. Genetic Analysis of Commercial Honey Bees from the Southeastern United States. *J.Econ. Entomol., 88*(5), 1216-1220.

Schiff, NM., & Sheppard, W. S. 1996. Genetic differentiation in the queen breeding population of the Western United States. *Apidologie, 27*, 77-86.

Sheppard, WS. 1989. A history of the introduction of honey bee races into the United

States, I and II. *Amer. Bee J.* 129: 617-619, 664-667.
Sherman, PW., Seeley, T. D., & Reeve, H. K. (1988). Parasites, pathogens and polyandry in social hymenoptera. *Am. Nat., 131*, 602-610
Taber, S. 1961.Forceps design for transferring honey bee eggs. *J. Econ. Entomol.* 54:247-250.
Tarpy DR, Seeley TD 2006 Lower disease infections in honeybee (*Apis mellifera*) colonies headed by polyandrous vs monandrous queens. 93: 195-199.
Seeley TD & Tarpy DR, 2007. Queen promiscuity lowers disease within honey bee colonies. *Proc. Royal Society of London,B.,* 274:67-72.
Wang D, Urisman A, Liu YT, Springer M, Ksiazek TG, Erdman DD, Mardis ER, Hickenbotham M, Magrini V, Eldred J, Latreille JP, Wilson RK, Ganem D, DeRisi JL. 2003. Viral discovery and sequence recovery using DNA microarrays. *PLoS Biology*.