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***In vitro* rearing of queen honey bees, *Apis mellifera* L.**

Ashley Mortensen¹, Jamie Ellis²

¹Co-PI: Department of Entomology and Nematology, University of Florida, Steinmetz Hall, Natural Area Dr., P.O. Box 110620, Gainesville, FL 32611-0620. mortensena@ufl.edu

²PI: Department of Entomology and Nematology, University of Florida, Steinmetz Hall, Natural Area Dr., P.O. Box 110620, Gainesville, FL 32611-0620. jdellis@ufl.edu

1. Introduction

A substantial amount of research has focused on determining the extent to which various pesticides, parasites and pathogens affect honey bee health (Chauzat et al. 2006, Higes et al. 2008, Le Conte et al. 2010, Neumann and Carreck 2010, VanEngelsdorp et al. 2010, Lebnh et al. 2013). However, experiments conducted within a honey bee hive are biased by many uncontrollable factors such as colony strength, weather conditions, and food availability (Hendriksma et al. 2011). Much of this bias can be overcome by rearing honey bee larvae in the laboratory (i.e. *in vitro*).

In vitro rearing methods eliminate colony stressors from the rearing environment of the developing honey bees (Crailsheim et al. 2012). Over the last 10 years, there have been significant improvements made to methods for rearing honey bee workers *in vitro* (Crailsheim et al. 2012, Schmehl et al. 2016). However, little focus has been directed towards the *in vitro* rearing of honey bee reproductives (queens and drones; Crailsheim et al. 2012). A method to rear queen honey bees *in vitro* could be used to investigate the impacts of pesticides and pathogens on queen development, physiology, and behavior.

Furthermore, a method to rear queens *in vitro* would allow queens to develop in an environment free of pests, pathogens, and pesticides that commonly challenge honey bee colonies. Optimized *in vitro* rearing conditions may result in queens that have increased fitness when compared to colony-reared queens due to queen relief from these common health stressors

during development. Therefore, we have built upon protocols established for workers in an effort to develop a reliable method to rear queen honey bees *in vitro*.

Aim 1: Develop a method to rear queen honey bees in vitro.

We modified the Schmehl et al. (2016) worker rearing protocol to develop our queen rearing protocol. We tested a total of 78 combinations of variations in the diet composition (Asencot and Lensky 1985, Rembold and Lackner 1981), feeding schedule (M. Simone-Finstrom, personal communication, January 2016), feeding volume (M. Simone-Finstrom, personal communication, January 2016), juvenile hormone III (JH) application (Asencot and Lensky 1984), method of JH application (Asencot and Lensky 1984), and pupal environment on 2,793 individuals.

Our most successful protocol yielded an overall survival rate to adult emergence of 66.67%. Of those individuals that survived, 81.3% emerged at 18 days old and had queen-like external characteristics (increased abdominal size and no corbiculae). These individuals were fed the Schmehl et al. (2016) A, B, and C diets in a modified feeding schedule (Table 1). Additionally, 10 μg of JH were incorporated into the diet when larvae were four to five days old (day 4 after grafting). As each larva consumed all of its food (typically day 6 or 7 after grafting), it was transferred individually from the larval development plate to the pupal development plate. All other parameters of the rearing process were consistent with those described by Schmehl et al. (2016).

In our second most successful protocol, 62.3% of the emerging adults were queen-like. These larvae were grafted onto a mass provisioning of 270 μl of Schmehl et al. (2016) diet A, and did not receive any supplemental JH (Table 1). Our production rate of 62.3% queen-like individuals is a notable increase from rates reported previously. In previous studies, a range of 0 - 44.4% of the adults that emerged developed into queens without supplemental JH (Buttstedt et al. 2016).

We did not dissect the resulting queen-like individuals; therefore, we are unable to confirm that we produced queens rather than pseudo-queens. Further replication of our method is necessary to determine if our protocol produced queens or pseudo-queens and if the protocols can be repeated consistently with the same level of success. We plan to repeat the study in spring 2017 and will perform confirmatory dissections on emerging adults.

Additional benefits of our queen *in vitro* rearing methods are that; 1) each queen is individually reared, 2) each queen is fed a known volume of diet, and 3) each queen consumes all of that diet. Previously published methods rear queens by allowing a group of larvae to feed *ad libitum* on a dish of diet that is not fully consumed (Figure 1; Buttstedt et al. 2016), making it difficult to use these protocols in studies involving pest, pathogen, and/or pesticide risk assessments. Our method allows for developing queens to be exposed to known volumes of pesticides and/or pathogens in a controlled environment, which has not been possible previously.

Table 1. Feeding schedule used in our most (81.3% queen-like individuals) and second most successful (62.5% queen-like individuals) *in vitro* queen rearing trials and the previously published larval rearing methods (Schmehl et al 2016).

Day	Most Successful Queen Protocol (supplemental JH)		2nd Most Successful Queen Protocol (no JH)		Previously Published Larval Protocol	
	diet	volume (μ l)	diet	volume (μ l)	diet	volume (μ l)
Graft	C	65	A	270	A	20
1	C	35	-	-	-	-
2	C	35	-	-	B	20
3	B*	35	-	-	C	30
4	A	35	-	-	C	40
5	-	-	-	-	C	50

*Denotes the addition of 10 μ g of JH to the larval diet

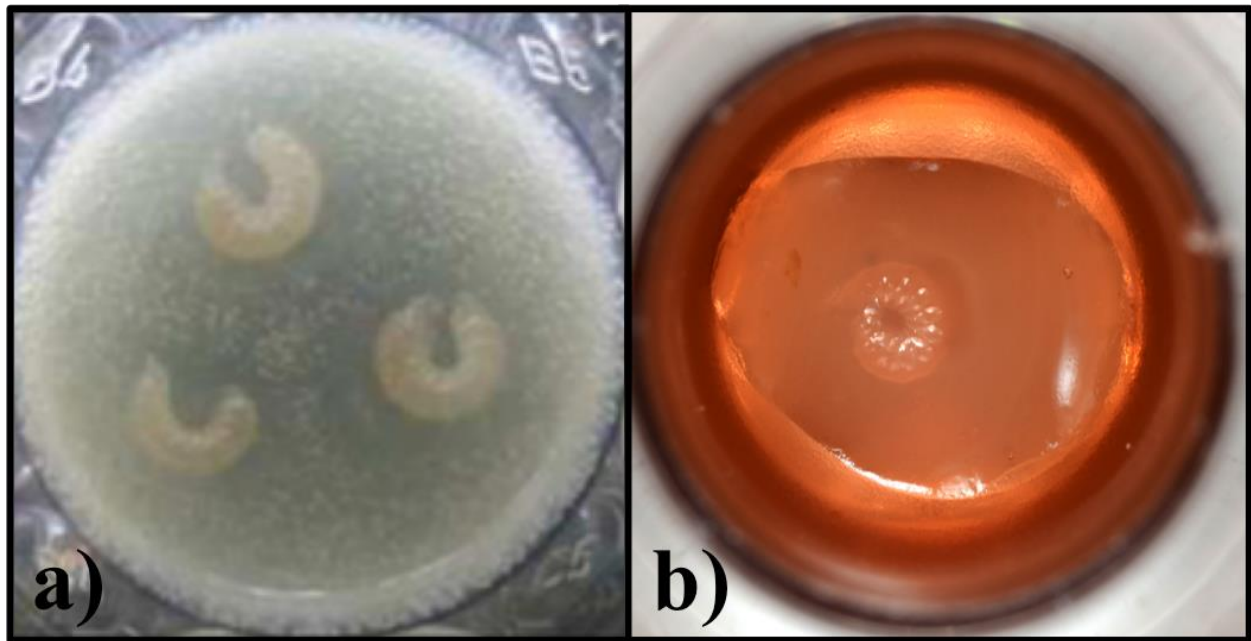


Figure 1. Visual comparison of methods for rearing queens *in vitro*. a) The previously published, group rearing conditions (Buttstedt et al. 2016). Note the three larvae present in the same well feeding on a large bolus of diet. b) Our new solitary queen rearing protocol. Note the single larva present in the well with a discrete amount of diet available.

Aim 2: Assess the vigor of the resulting adult queens.

It is imperative to determine if adult queens that have been reared *in vitro* are comparable to colony-reared queens. Unfortunately, our most successful trials with high rates of queen differentiation occurred after the 2016 mating season had ended. However, we did have a

moderately successful trial in late June that resulted in emerged queen-like adults prior to the end of the regional mating season.

Larvae in the late June trial were mass provisioned with 270 μ l of Schmehl et al. (2016) diet A (no JH application), and 59.6% of the surviving adults emerged on day 18. A subset of the queen-like individuals from this trial were confirmed to lack corbiculae. The remaining queen-like individuals (15 individuals) were introduced into mating nucs and allowed to attempt mating flights. Mating nucs were inspected 10, 14, and 21 days after introduction of the *in vitro* reared virgin queens. None of the *in vitro* reared queens were identified in the mating nucs at these inspections. However, three of the 15 mating nucs did have cells in which multiple eggs had been laid. It is possible that these eggs were laid by laying workers. Alternatively, a newly mated *in vitro* reared queen could have laid these eggs (it is common for young queens to lay multiple eggs per cell initially). We allowed the bees in the mating nucs to rear the brood to determine if the eggs were laid by a worker (haploid and would develop into drones) or a mated *in vitro* reared queen (diploid and would develop into workers). However, all mating nucs failed prior to capping any brood. Further field observations of *in vitro* reared queen viability are needed.

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