Investigation of an Ethanol Containing Diet for *Apis mellifera* Larvae

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Introduction

The long-term goals of this lab involve investigation of the developmental effects of ethanol on chemosensory behavior using a honeybee (*Apis mellifera)* model. To effectively study this relationship, ethanol must be administered during development without causing high rates of mortality. Since honeybee larvae lay in a pool of the food they ingest, there is a risk that an ethanol-containing diet will cause external drying effects. This could effectively kill the larvae even without their consumption of the diet. Therefore, the aim of this study is to investigate the survival rate of honeybee larvae exposed to an ethanol-containing diet. Ethanol concentrations of 0%, 2.5%, 5%, and 7.5% will be used as they are known to result in the presence of ethanol in the hemolymph of adult honeybees as well as impact behavioral outcomes.

Larval rearing protocols generally aim for a survival rate of at least 85% after 48 hours and 70% overall adult emergence (Schmehl et al., 2016). The aim of the present study is to modify techniques that have attained, or surpassed, this level of survival while adding ethanol into the development of the diet. Results will provide survivability information about the diet at several ethanol concentrations, allowing for future studies that either modify the diet further (if survival is lower than desired) or move on to other stages of development (if survival meets standards).

Materials and Methods

General Protocol

- Larvae will be collected from two hives located at the Robert A. Macoskey Center.
- Tools will be sterilized under a UV light before taking them to the field for grafting.
- Surfaces used for grafting and feeding will be cleansed with a 70% ethanol solution prior to use to maintain an aseptic workspace.
- Larvae will be grafted into sterile 48-well culture plates containing the prepared diet and transported back to the lab for incubation (Figure 1).
- The incubator housing developing larvae will be kept at 35°C throughout rearing.
- Desired 95% humidity will be maintained via two rows of water cells in each plate.
- Subsequent feedings involve adding the new diet to any remaining diet in the cell by placing it along the interior surface of the cell, while not submerging the larva.
- Mortality is determined by daily monitoring of the larvae for deflation, flaccidity, or black spots. All dead larvae will be removed from the culture plate.

Figure 1. A) Chinese grafting tool used for transfer of larvae into sterile 48-well plates. B) Larvae are grafted into 48 well plates containing one of the 4 concentrations of ethanol infused diet.

Larval Age

- Honey-bees go through four stages of development: egg, larvae, pupa, and adult.
- After 48 hours spent in the egg stage, larvae enter day 0 of the larval stage (Figure 2), which is the targeted developmental stage for the initial grafting procedure. Only larvae that appear to at this developmental stage will be used for experimentation.
- Larvae develop over 6 days before being capped and transitioning into the pupal phase. Appropriate growth and development over days 1-6 will be monitored throughout this study.

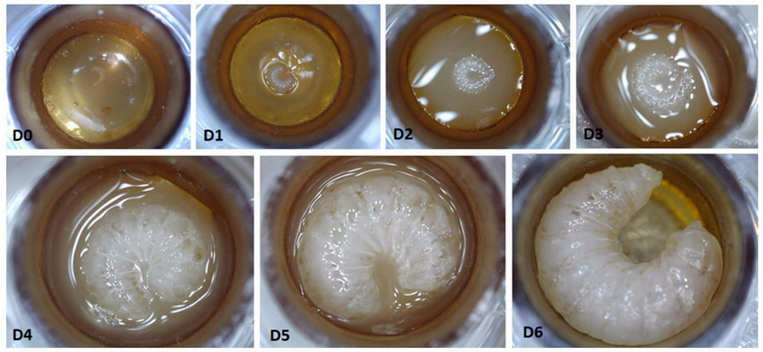

Figure 2. Standard larval development from day 0-6. (from Kruszakin & Migdal, 2022)

Diet

Bee larvae require protein, carbohydrates, minerals, fats, vitamins, and water for proper development (Collison, 2017). While these are normally derived from nectar and pollen, lab designed diets that mimic what occurs in nature have been previously developed (Schmehl et al., 2016). For the present study, three diets will be fed to the developing larvae. The components of each diet (Table 1) and amount allocated to each larva (Table 2) are adjusted based on larval age. The larvae will receive Diet A on day 1, no additional diet on day 2, Diet B on day 3, and Diet C on days 4, 5, and 6. Diets will be prepared the day of feeding, except for diet C, which will be made on day 4 and used for the remaining days. Prepared diet can be stored for up to four days at 4°C after creation. Stock diets will be divided into four and ethanol will be added to create each concentration for feeding.

	Amount of Diet Components (g)		
Diet Component	**Diet A**	**Diet B**	**Diet C**
Royal Jelly	1.661	1.613	9.375
Glucose	0.199	0.24	1.688
Fructose	0.199	0.24	1.688
Yeast Extract	0.034	0.0488	0.375
Water	1.661	1.613	5.625
Total	3.75	3.75	18.75

Table 1. Amount of diet components (g) required to feed approximately 150 larvae. The percentages for each diet were derived from Schmehl et al. (2016) and modified for the size of our experiment.

Day					
1	2	3	4	5	6
Diet					
A	N/A	B	C		
Amount of Diet (microliters)					
20	0	20	30	40	50

Table 2. Schedule of larval feeding over the six day grafting period. All four ethanol concentrations follow the same feeding schedule.

Discussion & Future Directions

Initial Timeline:

Queen bees may lay up to 250,000 eggs per year (Collison, 2017). Although egg production ceases over the coldest winter months, queens begin laying eggs again in early January even in northern climates. The rate of egg laying in the spring increases steadily until a peak of ~1500 eggs per day is reached in early summer (Collison, 2017). As the present study aimed to graft 72 larvae per round (18 per condition), egg laying rates were anticipated to allow appropriate larval samples to be taken while still leaving enough larvae behind for healthy colony growth. Thus, the initial intended schedule was to develop a diet during January, practice larval grafting in mid-February, and begin official data collection at the end of February through the month of March. As discussed below, issues with weather led to delays in this timeline.

Weather Considerations:

Weather conditions must be conducive for the collection of healthy larvae. Honeybees control the internal hive temperature to maintain a brood nest within a range of 33-36°C (91.4-96.8°F). Opening hives when the temperature is too cold can stress the developing brood. Eggs and larvae exposed to temperatures under 32°C (89.6°F) for too long develop into adults with physical, neural, and behavioral abnormalities (Collison, 2017). While many beekeepers report the ideal temperature for removing frames to be above 15.6°C (60°F), frames can be removed at temperatures above 10°C (50°F; Lee et al., 2020). Additionally, hives should not be opened during episodes of rain, as moisture is also detrimental to hive health (Lee et al., 2020).

While optimal weather conditions for opening hives are often met in Western Pennsylvania starting in late February, prompting the initial timeline for this study, this year has proven to be an exception. There have been very few instances in which the weather was conducive for collection of larvae. On the rare instances when weather was appropriate, not enough larvae at developmental stage 0 were present. Data collection will begin as the weather becomes more favorable in the upcoming weeks and, due to increases in egg laying into the summer months, can occur rapidly with multiple rounds of grafting being feasible within a short period of time

Future Directions:

- Failure of the study to reach the desired survivability rate will prompt modification of the diet with substitutions to counteract the drying effects of the added ethanol.
- Achievement of the desired survivability will permit further investigation including:
  - Collection of hemolymph of ethanol exposed larvae to determine ethanol concentrations (via gas chromatography)
  - Behavioral testing of bees exposed during larval development
  - Ethanol exposure during additional developmental stages (i.e., pupal only, larval + pupal) to determine survivability, hemolymph ethanol concentrations, and behavioral outcomes.

References

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