**Triglyceride and Storage Protein Production as an Indicator of Time Spent in Diapause**

**James T. Brown**

**MS Thesis Proposal**

**Advisor: Dr. Dan Hahn**

**Committee Member: Dr. John Beck**

**INTRODUCTION**

**Ecological Seasonality:**

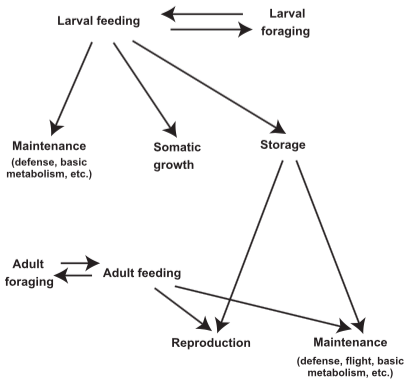
* What are seasons generally and how does its regularity or irregularity effect ecosystems and the organisms in those ecosystems
  + Talk about how phytophagous insects depend on timing their life cycles on food availability
* Talk about the confluence of life history timing and seasonality and resource availability
  + Why are seasons so important for insects to detect?
  + How can insects detect changes in their environment?...hormones
  + What effect does sensing these environmental changes have on the insect
    - Migration or diapause or both
      * Migration: Danaus plexippus is an example where both migration and a type of dormancy occurs. During autumn these animals mitigate the effects of their harsh environments through migration. To accomplish this migration, Monarchs migrate in a state of diapause, characterized by reduced responsiveness to reproductive and vegetative stimuli and increased lipid storage.
      * Diapause:
    - Finally discuss diapause as one of those choices

**Diapause:**

* In general, what is diapause
  + Programmed dormancy brought on by: temperature, light, or a combination of the two
  + Occurrence of diapause in across taxa
    - In Danaus plexippus migrating monarchs are in a state of reproductive diapause where
  + Protective state for when resources are low/ environment is inhospitiable
  + Hallmarks include reduced metabolism/respiration
  + Can be obligatory or facultative
* What does diapause look like in my model
  + Generally what do ECB face leading up to diapause
  + Cue that induce diapause
  + Life stage that diapause is signaled
  + Life stage that diapause is induced
  + Physiology of my system at diapause induction. Feeding, metabolism, respiration
  + End on a discussion about the proliferation of fat and proteins

**Fat Body:**

* How resource affect life histories of insects
  + CL Boggs: The schematic below is a resource allocation framework provided by CL boggs that does well to summarize the priorities of feeding stages of insects.



* What is the biology of the fat body
* The role of the fat body leading up to diapause
  + Lipid and protein production
* The overall goal of my project

The European corn borer lends its self to characterizing the role of the fat body in inducing diapause.

**European corn borer:** The life stragety of the European corn borer (ECB), ois to facultatively

**Thesis Objective**

**Hypothesis:** Given the understanding that UZ and BE strains of ECB are genetically different in how they regulate the length of diapause If triglyceride and storage protein levels play an important role in supporting the life history step after the larval wandering stage and given the additional metabolic demands of diapause. Could it be the case that these levels directly affect the length of diapause, such that larvae preparing to enter diapause and larvae preparing to molt into pupa will differentially express these products. Specifically, do UZ larvae preparing for their long diapause will produce more TG and SP to compensate for their longer diapause, while BE larvae preparing for their shorter diapause, produce less TG and SP.

“Studies detailing diapause-associated changes in intermediary metabolism and feeding physiology are needed across taxa with different diapause strategies to expand our understanding of the metabolic processes underlying prediapause reserve accumulation. The ultimate goal in this area is to under- stand the underlying neurological and endocrine signaling mechanisms that regulate diapause-associated shifts in feeding patterns and intermediary metabolism. ”

The objective of this study is to characterize the extent to which stored energy, more specifically the production of triglycerides and storage proteins, correlate with the incidence of diapause across the 4 phenotypes of European corn borer. Currently, more evidence is needed to better understand the energetic requirements of diapause incidence across taxa. While intuitively it may be logical to acquire more energy reserves in preparation for the energetic demands of diapause, this is not always the case (Hahn and Denlinger 2011). Thus, the univoltine-Z and bivoltine-E strains of European corn borer provides an excellent case study into how facultative diapause preparation is accomplished. So far information about the energetic requirements of diapause induction in ECB is limited to (…current knowledge about the ECB diapause preparation energetics…). While less is known about the link between phenotypic variation in triglyceride and storage protein production and the genotypic variation between the 2 strains. Specifically, quantifying the production of triglycerides and storage proteins produced by the fat body across the 2 strains of ECB is intended to approximate the amount of energy reserved for diapause as a function of length of diapause. Further quantifying the production of these reserves between diapause destined and directly developing larvae is intended to approximate the energy storage differential between diapause destined larvae and directly developing larvae.

I hypothesize that there is a direct relationship between the accumulated amounts of triglyceride and storage protein during the later portion 5th instar and larvae destined for diapause or direct development. Such that in the strain that larvae destined for a longer period of diapause will reserve more energy than those destined for a shorter diapause. Further, those larvae that are diapause destined will differentially reserve more energy than those larvae that are destined for direct development. To evaluate this hypothesis, I intend to quantify and qualify the production of triglycerides and storage proteins at the point in the ECB life history where triglyceride and storage protein production is at its peak.

European corn borer was chosen as the model for these experiments due to their facultative diapause life history strategy, differing genotypes and physiologies, and their different phenotypes. When either strain is exposed to the same photoperiodic and thermal cues in the laboratory, their specific response can be reproducibly observed but the physiological link between genotype and phenotype has not yet been described.

The evidence produced from this study will have two effects. First, it will add to the body science relating to the physiological requirements leading up to diapause in related taxa. Second, characterizing the production of triglycerides and storage proteins and how that production differs between the different phenotypes is something that has yet to be capitulated in *Ostrinia nubilalis*. Further, having a clearer understanding of the physiological requirements of this tenuous life history decision could have reaching effects on how we manage them as pests.

The first step will pertain strictly to preliminary trial information gathering. I will use this time to flush out problems with equipment and protocols. I will look for alternative ways to complete experiments and find and use all of that to inform the experimental aspects of my project.

**PROPOSED METHODOLOGY**

**Origin and Husbandry of European Corn Corer.** The univoltine and bivoltine strains of European corn borer we provided courtesy of (\_\_\_\_). The laboratory reared colonies were established in the year (\_\_) as larvae. These larvae have been sibling mated to over the course of (\_\_) and throughout the course of these experiments. Three environmental regimens implemented to accommodate the different requirements of each of the phenotypes. The cohort of individuals, from both strains, used to perpetuate the colony were reared using 16 hours of light and 8 hours of dark at a temperature of 26° C. Those that are to be used as sampling For the duration of these experiments, these colonies were maintained under stable hour light, temperature, and humidity regimens

**Preparing Wandering Larvae.** Sampling larvae when they have produced the ultimate amounts of both triglycerides and proteins will be crucial to accurately characterizing the differences in life history choice. For the purposes of these experiments that ultimate developmental period, the “wandering” stage, will be the point at which samples will be taken from the cohort of larvae. Because there are no other diagnostic characters that indicate a larva will be considered in the “wandering” when all the contents of the gut are cleared. Samples will be selected on day 15 that appear to be in the 5th instar, those larvae will be separated into individual arenas where they will continue to grow and feed for the following 4 days. Upon reaching day 4 the individual arenas will either be cleaned or the larva will be moved into a cleaned arena to continue developing until such point that it does not expell frass over the course of 8 to 12 hours, and thus is in the wandering stage.

Wandering larvae will be divided into two groups for triglyceride or protein analysis. Those larvae destined for protein analysis will be bled through a small incision where approximately 10-12µL of hemolymph will be extracted and used for protein analysis. Those larvae destined for triglyceride analysis will be lyophilized for approximately 48 hours and stored for triglyceride analysis.

**Triglyceride Extraction and Derivatization.** The larvae in the triglyceride group will be extracted in sets of 16. Two larvae from each treatment group, UZ16, BE16, UZ12, and BE12, will be paired with an “empty” tube that does not have a larva but is treated in the same way as the tubes with larvae. These “empty” tubes will function to describe the background effect of the extraction method. To monitor the efficiency of the extraction methodology, undecanoic acid will serve the function of a spike lipid. One larvae and its “empty” counterpart will receive the spike, while the other larvae and its counterpart will not. Each larva will be weighed after lyophilization to obtain a dry weight. A modified version of the Folch method to extract the total lipid content from each sample. To reduce the effects of oxidation, the remainder of the experiment will be performed on ice to reduce oxidation and 0.5mg/ml of BHT (butylated hydroxytoluene) will be added to methanol. Grinding beads (1.1-1.4mm Zirmil.2) will be added to each tube, mechanically homogenized for 30 seconds using a (\_\_\_), BHT in methanol will be added to the tubes, again homogenized for 30 seconds, then hexane will be added to the tubes and the tubes will be homogenized for a third and final time for 30 seconds. After grinding the sample, the next step will be to separate the lipid containing fraction from the rest of the insect matrix. Separating the phases requires the sample be centrifuged at 4°C for 5 minutes, the organic fraction containing lipids is collected, the samples will then be re-suspended in organic solvent and placed on a table shaker for 15 minutes. The process of centrifugation and table shaking will be repeated three times. After the organic layers have all been pooled, the pooled fractions will then be dried using a 0.9% solution of NaCl and that aqueous layer will be removed and the dry pooled fractions will be dried under a stream of N2 to remove the organic solvent from the lipids. Those “dry” lipids will be weighed. The pooled fraction of lipids extracted represents the total lipid content from the insect. To remove some of these additional classes of lipids the pooled, dried lipid fractions will be re-suspended in 4mL of organic solvent and passed through a SPE (solid phase extraction) cartridge. This cartridge will capture the non-target lipids and allowing triglycerides to pass through the matrix and be collected. Once non-target lipids have been removed from the solution dried under N2 and weighed, the triglycerides will be converted into a FAME (fatty acid methyl ester). To accomplish this conversion, the clean lipid solution needs to

**Triglyceride Analysis.**

**Protein Extraction and Quantification:** The larvae in the proteins group will be extracted in groups of 7. Collecting the lymph fluid will be done through a small incision into the cuticle at the tip pf the prolegs. Lymph fluid will be dotted onto stretched Parafilm wax where it is collected using a pipette tip. Lymph will be collected into a microcentrifuge tubes held on ice to reduce oxidation of the lymph. Each tube will contain 200µl of 1x PBS to simulate biological conditions and 3µL of Halt™ Protease and Phosphatase Inhibitor Cocktail to inhibit proteolytic enzymes. Protein quantification will be accomplished using the Bradford protein assay. Dilutions from each sample will be made into 1:1, 1:2, and 1:10 solutions This colorimetric assay is based upon mechanics of the protein in solution binding to the dye. As the dye and protein interact, there is a shift in the color of the dye from its unbound color of reddish brown, to blue, a color which deepens as more protein interacts with the dye.

**Protein Separation and Identification:**

**Data Analysis.**

Lyophilization of larvae:

**Refercences**