**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
    - Organisms deal with fluctuations in their environment to maintain homeostasis and to be competitive. Temperature is a ubiquitous stress that most all organisms actively regulate against. Temperature variance can be environmentally gradual, seasons that change from warmer to cooler as the year progresses, days that become warmer then cooler as the sun rises and falls. This type of cyclic temperature stress is predictable and organisms must synchronize their life histories to compliment theses stress cycles. can that pattern use to make life history decision.

To be competitive in one’s environment organisms must be able to utilize resources when they become available. Integrating predictable cues from the environment, organisms have evolved life history strategies that include ways to deal with these predictable cycles of resource abundance and resource scarcity. When resources are not around

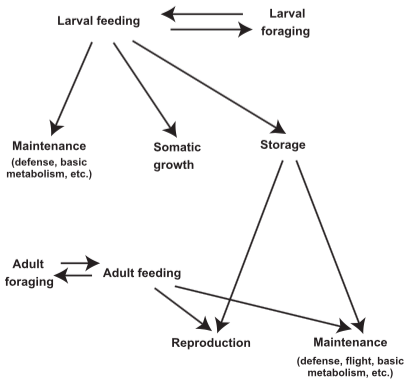
* + - Of the diapausing insects that do not feed after diapause must be able to not only survive the period of diapause but they must also be able to undergo adult pupal metamorphosis. Some insects prepare for this
* Talk about the confluence of life history timing and seasonality and resource availability
  + 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), is to facultatively

**Thesis Objective**

While intuitively it may seem that meeting the additional physiological stress of diapause, organisms should necessarily store more energy when preparing for that stressful time. However, other strategies have been documented (Hahn and Denlinger 2011). “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 will be to quantify and compare the difference in stored energy between two genotypically different strains of *Ostrinia nubilalis,* the European corn borer. Further, European corn borer (ECB) destined for and ECB avoiding diapause will be compared within each strain. Characterizing the energy stores of ECB destined for diapause, has yet to be capitulated in ECB and is necessary to understanding diapause biology and usefulness as a model system, managing it as an agricultural pest, and predicting its behavior as seasonal climates become less predictable (Denlinger 2008).

I hypothesize that the amount of energy a European corn borer stores in preparation for the additional stress of diapause, is related directly to how it performs during diapause. Specifically, diapause destined individuals preparing for diapause will increase their storage of triglycerides and storage proteins in direct relation to the length of time they will spend in diapause. The ECB strain preparing for a long period of diapause will store more energy than their shorter diapausing and diapause avoiding counterpart. Diapausing ECB may be storing energy in different ratios than diapause avoiders. Higher levels of triglycerides may be used to supplement water and protect against desiccation while higher protein stores could be used to rebuild damaged or depleted enzymes.

European corn borer model is well suited for this type of study. Within the species there are of 2 distinct genotypes that differentially express the diapause phenotype. The univoltine-Z (UZ) genotype expresses a long diapause phenotype, while the bivoltine-E (BE) genotype expresses a shorter diapause phenotype. Further, the expression of the diapause phenotype is facultative. When exposed to a photoperiod of 12h:12h (light hours:dark hours) the diapause phenotype is expressed, photoperiods of 16:8 suppress the diapause phenotype. The strains of ECB persist as inbred siblings, originating from naturally occurring populations genotype can either express or avoid. ECB preparing for a longer period of diapause will store more energy in the form of fats and protein, while ECB preparing for shorter period of diapause should store relatively less energy in the form of fats and proteins. sthere is a direct relationship between the amount of energy stored when feeding ends and the length of time an individual spends in the diapause state. Such that ECB destined for a longer period of diapause will store more energy than ECB destined for a shorter diapause. Further, the larvae destined for diapause will differentially store more energy than those larvae that are avoiding. To that end I will

Characterizing these metabolic intermediates is intended to approximate the amount of energy an individual has reserved after feeding ends. 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.

Approximately, $10 billion dollars is spent annually on chemical insecticides to control the damaging effects of insect pests (Pimentel 2005). Corn is an incredibly valuable crop in the United States and protecting it from actively feeding phytophagous insects includes the dynamic use of chemicals and biotechnology.

To control the ephemeral outbreaks of ECB, farmers in the US spend approximately $10 billion dollars on chemical pesticides.

Mitigating the persistent threat to this crop is accomplished through genetically engineered corn seed expressing an insect feeding toxin found in *Bacillus thurgensis* (BT)*.* In 2016, 92% of the corn acreage in the US was planted with BT corn. This type of pest management is a very powerful tool due to its specificity for phytophagous insects. However, the widespread use of this toxin pressurizes competition in the population. Those individuals in that can survive the toxic effects are given a mating advantage over its less advantaged, or dead, peers. Combined with the ability of ECB to produce one or two large generations a year, resistance across populations can be quickly amplified.

Investigating the physiological requirements of this tenuous life history decision will expose diapause phenology to being controlled. Strategies that can precisely affect the progression of the ECB through diapause could be valuable. Perturbing the corn borers ability to survive diapause by effecting how it allocates resources could be used as an added layer of pest management.

**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 (Gelman and Hayes 1982). Staging proper wandering samples will be done using individual rearing arenas. Larvae will be separated into individual arenas around the time of their 5th instar molt and given food over the next few days. Once the larvae reach day 5, they are observed for wandering approximately every 8 hours. During these observations, the arena will be cleared of frass, when the arena no longer needs to be cleared will indicate the start of the wandering stage.

Wandering larvae will be divided into two groups; triglyceride analysis 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 stored for protein analysis. Those larvae destined for triglyceride analysis will be lyophilized for approximately 36-48 hours and stored for triglyceride analysis.

**Triglyceride Extraction and Derivatization.** The larvae in the triglyceride group will be sampled in cohorts of 16 using polypropylene microtubes, eight samples with larvae and eight samples without larvae. Larvae from each treatment group (UZ16, BE16, UZ12, and BE12) will be paired with samples without larvae, blanks. These blanks will be treated in the same way as the larva samples and function to describe the background effect of the extraction method and associated materials. To monitor the efficiency of the extraction and derivatization methodology, (\_Tri-Mix?\_) will serve the function of a spike standard. Half of the larvae and half of the blanks will receive the spike, while the other larvae and blanks will not. Each larva will be lyophilized until they lose no more than 1% of their body weight in a 24 hour period. These dry larvae will be weighed, and a modified version of the Folch method will be used to extract the total lipid content from each sample (Folch et al. 1957). To reduce the effects of oxidative damage, the remainder of the triglyceride extraction will be performed on ice and a solution of 0.5mg/ml of BHT (butylated hydroxytoluene) will be prepared fresh before each extraction. Each sample will be mechanically homogenized for 30 seconds using media grinding beads (1.1-1.4mm Zirmil.2) added to each tube and a Precellys®24 bead beater. 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 (Gelman and Woods 1983). 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**

Objective

1. Pimentel, D. 2005. ‘Environmental and Economic Costs of the Application of Pesticides Primarily in the United States’. Environment, Development and Sustainability. 7: 229-252.
2. Hyde, J., M. Martin, P. Preckel, and C. Edwards. 1999. The Economics of Bt Corn: Valuing Protection from the European Corn Borer. Applied Economic Perspectives and Policy. 21: 442-454.
3. United States Department of Agriculture Economic Research Service,. 2017. Recent Trends in GE Adoption. Seth J. Wechsler.
4. DENLINGER, D. 2008. Why study diapause?. Entomological Research. 38: 1-9.

Husbandry and sampling

1. Gelman, D. and D. Hayes. 1982. Methods and Markers for Synchronizing Maturation of Fifth-Stage Larvae and Pupae of the European Corn Borer, Ostrinia nubilalis (Lepidoptera: Pyralidae). Annals of the Entomological Society of America. 75: 485-493.

Lymph collection

1. Gelman, D. and C. Woods. 1983. Haemolymph ecdysteroid titers of diapause- and nondiapause-bound fifth instars and pupae of the European corn borer, Ostrinia nubilalis (Hübner). Comparative Biochemistry and Physiology Part A: Physiology. 76: 367-375.

Extraction and Derivatization

1. Folch, J., M. Lees, and G. Stanley. 1957. A SIMPLE METHOD FOR THE ISOLATION AND PURIFICATION OF TOTAL LIPIDES FROM ANIMAL TISSUES. Journal of Biological Chemistry. 226: 497-509.