Experiment 1: Estimating the Onset Diapause and Classifying Diapause Programmed Larvae Using Metabolic Activity

Introduction

Comparing stored nutrition between each genotype was contingent on sampling larvae at developmentally similar growth stages and required characterization of the diapause ontogeny of each diapause genotype. Diapause programming in most insects suppresses metabolic activity, supresses development, and interrupts feeding. European corn borers diapause as larvae at the end of the last larval instar feeding stage, a so-called wandering larval diapause. After diapause, larvae resume normal development, first pupating and then eclosing as adults.

Materials and Methods

I tracked the developmental stages of individuals exposed to diapause-inducing and non-diapause treatments for forty days starting on day one of the last larval instar. Each day, larvae were observed and I recorded the developmental phenotype of each individual and its "diapause" status. Within each genotype, diapause was determined by comparing development of individuals reared in each treatment as outlined below.

Larvae exposed to the non-diapause treatment eventually pupated. The timing of pupation in the non-diapause treatment was used as a developmental time point to mark the end of the larval stage. Diapause programmed individuals that remained larvae after the time that all the non-diapause individuals pupated were assumed to be in diapause. Diapause programmed larvae that pupated after diapause onset but before the end of the 40-day trial were classified as "shallow diapause" individuals, and diapause-programmed larvae that did not pupate during the 40-day trial were recorded as "deep diapause" larvae.

CO2 production of larvae programmed for diapause was measured to determine the degree to which metabolic activity differed between each diapause genotype and differed between shallow and deep diapausing individuals. Beginning on day one of the last larval instar, mass was measured and recorded CO2 production every day. Before measuring CO2 production, larvae were isolated into airtight chambers free of CO2. Each chamber was designed from plastic Air-Tite 5mL Norm-Ject luer tip syringes (product A5) fitted with a three position stopcock. To produce CO2 free air when sealing the insect into the respiration chamber, atmospheric air was pumped and bubbled through water with a pH of 4. The CO2 free air was then pumped into the chamber to replace atmospheric air initially sealed in the chamber with the larva that may have contained some environmental CO2. Larvae were then held in these chambers for approximately one hour. After the hold time elapsed, each sealed chamber was attached to a Licor gas analyzer (model LI6262) to quantify the CO2 produced by each larva. These data were visualized using Expedata software.

The day mass peaked in individuals with the short-diapause genotype was used to compare CO2 production between shallow diapause larvae and deep diapause larvae at an equivalent developmental time point. After measuring and comparing CO2 production, there was no significant difference between shallow diapause and deep diapause larvae and I was unable to discriminate or remove shallow diapause larvae from deep diapause larvae within the short-diapause genotype.

Experiment 2: Determing wandering stage and sampling larvae for lean mass and lipid content.

To determine the onset of the wandering stage and approximate peak nutrition stores, I assayed larvae during the final larval instar for wandering by isolating larvae from diet and tracking their frass production. Larvae were removed from artificial diet and held in isolation for thirty minutes. After thirty minutes of isolation, larvae that did not produce frass were recorded as "wandering". Using this wandering assay, I tracked larvae daily and recorded following developmental events: 1) the day that larvae eclosed into the final instar, 2) the wandering day - as defined as the day they stopped producing frass, and 3) pupation. Wandering day was used to mark the end of larval development across treatments and to approximate the onset of diapause among larvae programmed for diapause. Wandering day was determined for each larval sample using this assay. Larvae samples intended for lean mass and lipid measurements were only sampled once and larvae determined not to be wandering were removed from the experiment.

To investigate the relationship between nutrition and the genetically determined diapause length, nutrient stores in diapause programmed larvae were measured before the onset of diapause and during diapause. Diapause programmed larvae were sampled on the first day of the final larval instar and on the wandering day of the final larval instar to capture the peak of lipid storage before the onset of diapause. To capture the rate of nutrition decline during diapause, diapause programmed larvae were sampled at 15, 20, and 30 days after the wandering stage.

At each sampling time point each larva was assigned a unique identifier and freeze-dried under vacuum to remove water. Samples were determined to be sufficiently dry when their weight varied by less than 1% over a 24-hour period, and their dry mass was recorded. Dry larval samples were then assigned to an experimental cohort and stored in a -80℃ freezer. Each experimental cohort consisted of larvae from each biological cohort. Individuals from each experimental cohort were then measured for lipid mass. To measure lipid mass, lipid content from each larva was extracted using a slightly modified Folch method (Gossert 2011). Larvae samples were solubilized in a 3:1 solvent mixture of hexanes and methanol and lipids were removed and collected. The density differences between hexanes and methanol facilitates physical separation between the two solvents and produces two liquid layers when mixed together. The polarity differences between the two solvents facilitates the selective solubility of molecules into each layer. Proteins and other charged molecules have polarities similar to methanol and readily solubilize into the denser methanol layer while lipids and other neutral molecules that have polarities similar to hexanes readily soluabilize into the less dense hexanes layer. The less polar hexanes layer containing the neutral lipids was siphoned away from the methanol layer. Finally, the hexanes layer was dried away, concentrating the lipids and allowing for them to be quantified gravimetrically.