CHAPTER 3 EUROPEAN CORN BORER: THE RELATIONSHIP BETWEEN STORED RESOURCES AND DIAPAUSE TIMING

3.1 Background

According to the National Oceanic and Atmospheric Administration, 2016 was the warmest year on record and temperature increases are expected to continue through the year 2100 [25, 26, 27]. As seasonal temperatures increase, warm summers will expand, cool winters will contract, and temperatures during the spring and fall will become less predictable [28, 29]. Animals monitor variation in seasonal factors like temperature and photoperiod (daylight hours) because these factors can affect the availability of nutrition, mates, and habitat. Seasonality predictably cycles between conditions that are favorable for insect activity and conditions that are stressful and unfavorable. Many temperate-dwelling insects protect themselves from seasonal stress by entering diapause before their environment becomes unfavorable [1].

Insects in diapause can survive for months exposed to harsh conditions and typically do so without access to nutrition by lowering their metabolic activity and suspending their development [30, 3]. Before the environment becomes unfavorable, insects prepare for diapause by accumulating and storing nutrients in the form of lipids, proteins, and carbohydrates [1]. For example, increased energy storage in the form of proteins has been reported in the Colorado potato beetles (L. decemlineata) (Kort and Koopmanschap 1994) and the southwestern corn borers (D. grandiosella) (Brown and Chippendale 1978), while increased lipid storage has been reported for the pink bollworm (P. gossypiella) (Adkisson et al. 1963) and Culex pipens mosquitoes (Mitchell and Briegel 1989), among others. Energy stores fuel insect metabolism during diapause, and after diapause these stored resources are redirected to accomplish post-diapause functions. However, metabolic activity for many insects is temperature dependent and insects preparing for diapause in warmer environments may struggle to meet the energy demands of an increased metabolism or possibly divert energy away from storage.

Insects entering diapause without adequate nutrition stores could run out of stored energy and exit diapause before winter ends leaving them exposed to low winter temperatures and mortality. A study using Calliphora vicina (Robineau-Desvoidy) as a model explored the effect of nutrition on the duration of diapause [44]. While preparing for diapause, the diet of some larvae was restricted and they found that when diet was restricted larvae entered diapause with less mass and remained in diapause for a shorter period than larvae given an unrestricted diet [44]. Insects that exit diapause early could be exposed to a stressful winter environment or they may not have enough stored nutrients and other metabolic substrates remaining to meet the anabolic requirements for post-diapause development, metamorphosis, repair, and other post-diapause activities like reproduction [3, 4].

Climate change could also decrease levels of stored nutrition as warmer and more variable fall and winter temperatures increase insect metabolic activity [31, 32, 33, 4]. Thompson and Davis (1981) showed that insects experiencing warmer and more variable temperatures at the beginning of diapause accumulate less resources before the onset of winter [42, 43]. These researchers reared diapausing Diatrea grandiosella Dyar moths in warm temperatures and compared lipid mass at the end of diapause to moths diapausing in cool temperatures. The moths that were exposed to the warmer temperatures demonstrated a significant decrease in lipid stores at the end of diapause compared to moths in cooler conditions [Thompson and Davis 1981].

Warmer temperatures during diapause preparation could increase metabolic rates and could redirect resources away from nutrient storage. Being unable to build up enough stored energy before the onset of diapause could limit an insects ability to enter diapause before the onset of winter. Similarly, warmer winter temperatures could also increase the metabolism of diapausing insects, causing them to deplete stored energy before environmental conditions become favorable for development the next spring, leading to mortality. Surviving diapause with reduced resources could also post-diapasue adults and limit critical functions like dispersal, mating, and reproduction.

Ostrinia nubilalis (European corn borer) is an excellent model to understand how warmer fall temperatures might influence nutrition storage ahead of diapause, as well as the role of warmer winter temperatures on energy depletion during diapause. European corn borer exists as at least two naturally segregating, genetically distinct strains with unique diapause genotypes. Regardless of genotype, these two strains can and do occur at the same latitude and experience the same fall and winter, however the diapause genotype of each strain expresses a specific length of diapause. Larvae with the "long-diapause" genotype experience a warmer, longer diapause because they enter diapause earlier in the fall and exit later in the spring. Alternatively, larvae with the "short-diapause" genotype experience a shorter, cooler diapause because they enter diapause later in the fall and exit earlier in the spring. Comparing nutrition storage strategies between these two strains could build our understanding of how insects might adjust to warming winter temperatures as Earth’s climate changes.

Warmer fall temperatures experienced by the two European corn borer strains could lead to increased metabolic activity and in turn increase the share of energy required to fuel their metabolism ahead of diapause. During diapause, both strains experience the same thermal environment and unless their metabolism is significantly influenced by diapause genotype, metabolic activity should be similar between the two strains. I predict the genotype that survives the longer, warmer diapause period will accumulate more nutrient stores prior to diapause compared to the genotype with a shorter larval diapause. During diapause, and regardless of diapause genotype, I expect that larvae will deplete nutrient stores at a similar rate. To investigate the relationship between diapause length and nutrient storage, lipid stores at the start of diapause and during diapause were measured. This research showed larvae with the long-diapause genotype accumulated more lipid mass at the onset of diapause compared to larvae with the short-diapause genotype, however the rate of lipid depletion during diapause was less conclusive.

As the Earth’s climate warms, some species of insects may find it difficult to adjust to warmer seasonal temperatures, experience reductions in population size or become extinct. European corn borers with the short-diapause genotype could provide an example of how climate might negatively impact insect populations if warmer diapause temperatures drain nutrient stores prematurely and these larvae exit diapause before seasons change. However, the effects of climate change for some insects could also be positive. If the effects of warmer diapause temperatures can be mitigated by larger nutrient stores then insects that utilize this strategy like long-diapause European corn borers could thrive.

3.2 Methods

3.2.1 General Rearing

*Ostrinia nubilalis* eggs were provided as a courtesy from Dr. Erik Dopman's laboratory at Tufts University. The two genetically distinct European corn borer strains used during my experiment were collected as a mixture of larvae, pupae, and adults from New York state prior to 2015 and kept as separate colonies. Strain identity was determined genotypically using the *pgFAR* autosomal gene, this gene codes for an important enzyme involved in determining the female sex-pheromone blend, and is partly responsible for maintaining strain differences. The *pgFAR-Z* is carried by the Univoltine-Z (UZ) strain and the *pgFAR-E* allele is carried by Bivoltine-E (BE) larvae and each allele produces a distinct pheromone blend. For the duration of the experiment, colonies of each genotype were mass reared at 26°C under a 16L:8D photoperiod to promote continuous development.

Individuals intended for experimentation were collected as eggs from each colony and organized into "biological cohorts". A biological cohort was defined as clutches of eggs oviposited on a single day by females of the same strain. Initially, eggs from each biological cohort were held under a 16L:8D photoperiod, 23°C and 65% rH until they hatched. Upon hatching each biological cohort was divided and reared in either the diapause treatment (12L:12D photoperiod, 23°C, and 65% rH) or the non-diapause treatment (16L:8D photoperiod, 23°C, and 65% rH). Larvae from each biological cohort were reared in mass and provided artificial ECB diet ad libitum, purchased from Frontier Agricultural Sciences (Product F9478B). As larvae from each biological cohort within each treatment reached the end of the fourth instar, they were separated and reared individually in 32-well bioassay trays purchased from Frontier Agricultural Sciences (Product RT32W). Each well of the bioassay tray was provisioned with diet and returned to its treatment conditions until sampling.

3.2.2 Experiment 1: Estimating the Onset of Diapause and Using Metabolic Activity to Classify the Intensity of Diapause Programmed Larvae

I tracked the developmental stages of individuals exposed to the diapause- and the non-diapause treatments for forty days starting on day one of the last larval instar. Larvae were observed daily and their developmental phenotype and diapause status were recorded. To determine the onset of diapause, the development of individuals reared in the non-diapause treatment was compared to larvae in the diapause treatment. Because larvae in the non-diapause treatment eventually pupated, the timing of pupation in the non-diapause treatment was used to mark the start of diapause for larvae in the diapause treatment. 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.

Carbon dioxide production and wet mass were measured starting on the first day of the last larval instar. To measure CO2 production, larvae were first isolated into airtight chambers designed from plastic Air-Tite 5mL Norm-Ject luer tip syringes (product A5) fitted with a three position stopcock. A single larva was placed into a chamber, atmospheric air that was bubbled through water with a pH of 4 to scrub CO2 from the air and humidify the airstream. This CO2 free air was then pumped into the respirometry chamber to replace atmospheric air in the chamber and finally the larvae was sealed into CO2 –freechamber. 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 wet mass peaked was used as a developmental timepoint to compare CO2 production between genotypes and between treatments.

3.2.3 Experiment 2: Determination the Onset of Diapause and Sampling Larvae for Lean Mass and Lipid Content

To determine the onset of diapause, larvae were assayed during the final larval instar for wandering, defined as the day larvae stop producing frass. First, 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 for up to forty days and recorded following developmental events: 1) the day that larvae eclose into the final instar, 2) the wandering day, and 3) pupation. Wandering is a developmental step that occurs in diapause programmed and continuously developing European corn borers and was used to mark the end of larval feeding and the start of larval diapause. All larval samples intended for lean mass and lipid measurements were assayed for wandering only once and larvae determined not to be wandering were removed from the experiment.

To investigate the relationship between nutritional stores and diapause length genotype, lean mass and lipid mass in larvae from each treatment before at the onset of diapause and during diapause for diapause programmed larvae. All 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 mass and lean mass before the onset of diapause. To capture the rate of nutrition decline during diapause, diapause programmed larvae were sampled 15, 20, and 30 days after they reached the wandering stage.

Sampled larvae were assigned a unique identifier and freeze-dried under vacuum to remove water. When the mass of each freeze-dried larvae varied by less than 1% over a 24-hour period, dry mass was recorded. After drying, 657 larval samples were then assigned to one of the 43 experimental cohorts and stored in a -80℃ freezer. Each experimental cohort consisted of larvae from each biological cohort. To measure lipid mass, lipid content from each larva was extracted using a slightly modified Folch liquid-liquid extraction method (Gossert et al. 2011). Larvae samples were solubilized in a 3:1 solvent mixture of hexanes and methanol and the hexanes layer containing the lipids were removed and collected. Finally, the hexanes layer was dried away from the lipids and the methanol layer was dried away from the insect lean mass and each was quantified gravimetrically.

3.2.4 Statistical Analyses

All statistical analyses were performed using R studio software. In experiment 1, diapause status was measured in 100 larvae for 40 days. The percentage of individuals in diapause was calculated daily as the number of individuals that pupated divided by the total number of individuals alive during each observation day (larvae and pupa). Wet mass measurements and CO2 production were taken in 100 individuals and analyzed using a linear model. Wet mass, CO2 production, and day of peak mass, were included as fixed factors (3-1).

In experiment 2, I calculated the day of wandering as the total number of days between eclosion into the final larval instar and the day frass production ended. The day that frass production ended for the majority of larvae was determined to be the wandering day. Lipid stores were measured in 266 individuals and analyzed using a linear mixed effects model. The statistical model included: lipid mass, diapause genotype, and treatment as fixed effects, diapause genotype and treatment were interacting fixed effects, and lean mass was a covariate. Biological cohort was also included in the linear model as nested within experimental cohort, and experimental cohort was as a random factor (3-4)(3-8). Lean mass was measured in 338 individuals and analyzed using a linear mixed effects model. The statistical model included: lean mass, diapause genotype, and treatment as fixed effects, diapause genotype and treatment were interacting fixed effects. Biological cohort was also included in the linear model as nested within experimental cohort, and experimental cohort was as a random factor (3-2)(3-6).

3.3 Results

3.3.1 Experiment 1: Metabolic Activity

Individuals in diapause programming conditions were considered to be in deep diapause if they remained in the larval stage throughout the 30-day trial larvae that pupated before the end of the trial were labeled shallow diapause. Long-diapause genotype larvae responded to diapause programming as expected with deep diapause reported in 100% of individuals (3-1). Despite being reared in diapause programming conditions, only 33% of short-diapause genotype larvae were in deep diapause while 66.6% were recorded to be in shallow diapause by the end of the 30-day trial period (3-1).

In an effort to separate shallow from deep diapausing individuals with in the short-diapause strain before the onset of wandering wet mass was tracked in individuals from each diapause genotype and treatment. On the day wet mass peaked, differences in the timing and the accumulation of wet mass between non-diapause larvae, deep-diapause larvae, and shallow-diapause larvae was compared. In the non-diapause treatment, long-diapause genotype individuals peaked in mass on day 5 and short-diapause genotype larvae peaked in mass on day 3 (3-4A). In diapause-programming conditions, mass peaked in long-diapause genotype larvae on day 9 and short-diapause genotype larvae peaked in mass on day 6 (3-4B). To capture the relationship between treatment and metabolic activity I compared CO2 production of diapause programmed individuals to non-diapause individuals of the same diapause genotype (3-6). I found diapause-programmed individuals produce significantly less CO2 compared to their non-diapause counterparts (long-diapause genotype: t-value=4.50, Df=30, p-value<0.000; short-diapause genotype: t-value=5.00, Df=43, p-value<0.000)(3-1C,3-1D). Additionally, I compared CO2 production between long-diapause and short-diapause individuals reared in the same conditions (3-5). I found that long-diapause individuals had lower CO2 production than short-diapause larvae, regardless of rearing conditions (diapause programming: t-value=-5.51, Df=26, p-value<0.000; non-diapause: t-value=-3.74, Df=47,p-value<0.001)(3-1A,B). CO2 production was also compared between shallow-diapause larvae and deep-diapause larvae within the short-diapause genotype (3-7B). I found no significant difference in CO2 production between shallow-diapause individuals and deep-diapause individuals (t-value=-1.03, Df=14, p-value=0.319) (3-1E).

3.3.2 Experiment 2: Stored Lipids

At the start of the wandering stage, these larvae cease feeding and I used this diagnostic to approximate the peak of nutrient accumulation. The wandering day was calculated as the number of days needed to reach the wandering stage after eclosion into the final larval s (lean mass: t-value=-9.70, Df=133.3, p-value<0.000; lipid mass: t-value=-10.23, Df=191.6, p-value<0.000) (3-3B),(3-5B). Additionally, long-diapause individuals in diapause programming and non-diapause conditions had greater lean mass and bigger fat stores compared to short diapause genotype individuals (lean mass:t-value=6.85,Df=10.9, p-value<0.000; lipid mass: t-value=4.08, DF=186.8, p-value<0.000) (3-8),(3-3B),(3-5B). To assess whether the long-diapause and short-diapause genotypes differed in their utilization of their nutrient stores during diapause, fat stores and lean mass were also measured 15, 20, and 30 days after the onset of diapause (3-9). Long-diapause individuals had significantly more lean mass at the onset of diapause than short-diapause larvae (t-value=2.45, Df=10.7, p-value=0.033) (3-7A). Long-diapause genotype individuals also had larger fat stores at the onset of diapause than short-diapause larvae (t-value=4.74, Df=16.7, p value=0.0002) (3-9A). However, within each diapause genotype lean mass and fat stores did not significantly decline during diapause (3-7B,C) (3-9B,C), with one notable exception. Fat stores among short-diapause individuals was significantly lower on day 15 compared to other days sampled during diapause from the short diapause population (t-value=-3.90, Df=111.4, p-value<0.000) (3-9C). While the data collected during diapause for lean mass and lipid mass depletion are sparse, they do point towards the possibility that the rate of lean mass depletion and fat store depletion may not be associated with diapause genotype.