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, the duration of 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 Colorado potato beetles (*L. decemlineata*) (Kort and Koopmanschap 1994) and 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 and possibly divert resources away from storage.

Insects entering diapause without adequate nutrition stores may exit diapause before winter ends leaving them exposed to an unfavorable environment and thereby increasing 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. The authors 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 stressful low winter temperatures 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 in diapausing insects 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 fat before the onset of winter [42, 43]. These researchers reared diapausing *Diatrea grandiosella* Dyar moths in warm temperatures and compared lipid mass to moths diapausing in cool temperatures. The moths that were exposed to the warmer temperatures also 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 insect’s 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 adults post-diapause 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 borers 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 the next spring. Alternatively, larvae with the "short-diapause" genotype experience a shorter, cooler diapause because they enter diapause later in the fall and exit earlier the next 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.

Adjusting to climate change for some insect species may be difficult because warmer seasonal temperatures could lead to reductions in population size or extinction. 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 f could also be positive for some insects. 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.

Warmer fall temperatures experienced by the two strains of European corn 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 rely on stored nutrients to fuel their suppressed metabolism and both strains experience the same thermal environment. Unless their metabolism is significantly influenced by diapause genotype, metabolic activity during diapause 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. However, 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 in each strain. This research showed that larvae with the long-diapause genotype accumulated more lipid mass at the onset of diapause compared to larvae with the short-diapause genotype. However whether the rate of lipid depletion between the two strains differed during diapause was inconclusive.

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* allele is carried by the Univoltine-Z (UZ) strain and the *pgFAR-E* allele is carried by Bivoltine-E (BE) strain larvae, and each allele produces a distinct pheromone blend. For the duration of the experiment, colonies of each genotype were 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-inducing 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 together in gorups and provided artificial European corn borer (ECB) diet ad libitum (Frontier Agricultural Sciences, Newark, DE, 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 (Frontier Agricultural Sciences, Newark DE, Product RT32W). Each well of the bioassay tray was provisioned with diet and returned to either diapause-inducing or non-diapause 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 non-diapause larvae eventually pupate, the timing of pupation in the non-diapause treatment was used to estimate the start of diapause for larvae in the diapause treatment. Diapause programmed larvae that pupated after the estimated onset of diapause but before the end of the 40-day trial were classified as shallow diapause individuals, and larvae that did not pupate during the 40-day trial were classified 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 respirometry chambers (Air-Tite, Virginia Beach, VA., product AL5) fitted with a three position stopcock. A single larva was placed into a chamber, atmospheric air 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 the atmospheric air in the chamber and finally the larvae was sealed into CO2 –freechamber. Larvae were then held in these chambers for approximately 1 hour and the exact hold time each individual larvae was recorded. After the hold time elapsed, each sealed chamber was attached to a gas analyzer (Li-cor, Lincoln, NE., model LI-6262) to quantify the CO2 produced by each larva. These data were visualized using Expedata software (Sable Systems International, Las Vegas, NV.). The day wet mass peaked was used as a clearly identifiable developmental timepoint to compare CO2 production between genotypes and treatments.

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

Stored energy was measured at the onset of diapause, because energy stores are at their peak at the start of diapause. I diagnosed the onset of diapause in final larval instar larvae by assaying for the termination of frass production, which signifies the start of the wandering stage. The wandering stage is a developmental step that occurs at the end of the larval feeding stage in continuous developing larvae and those programmed for diapause. 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 the population of 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. 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 were measured in larvae from each treatment. Larval samples were taken 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. To capture the rate of nutrition depletion 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 extraction cohorts and stored in a -80°C 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 (version 1.1.383). In experiment 1, diapause status was measured in 100 larvae for 40 days. The percentage of individuals in diapause was calculated on each observation day as the number of individuals that pupated divided by the total number of individuals alive (larvae and pupa). Wet mass measurements and CO2 production were taken for 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 for each sampled larva. 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 used as 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 extraction cohort, and extraction 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 post-feeding trial period. Larvae that pupated before the end of the 30-day trial period, but after the last of the non-diapause treatment group, were characterized as shallow diapause. Long-diapause genotype larvae responded to diapause programming as expected with deep diapause reported in 100% of individuals (3-1). Only 33% of short-diapause genotype larvae stayed in deep diapause while 66.6% showed a shallow diapause response by the end of the 30-day trial period, despite being reared in diapause programming conditions, (3-1).

In an effort to separate shallow from deep diapausing individuals within the short-diapause strain, wet mass was tracked in individuals from each diapause genotype and treatment starting on the day larvae eclosed into the final larval instar. On the day wet mass peaked, differences in the timing and the accumulation of wet mass between non-diapause larvae in the long-day treatment, as well as deep-diapause larvae and shallow-diapause larvae in the short-day treatment were 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). Within each strain, I compared CO2 production of diapause-programmed individuals to non-diapause individuals to capture the relationship between photoperiod and metabolic activity (3-6). I found diapause-programmed individuals produce significantly less CO2 compared to their non-diapause counterparts within each genotype (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 individuals with different diapause genotypes 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). Finally, within the short-diapause genotype CO2 production was also compared between shallow-diapause larvae and deep-diapause. (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 and Lean Mass

The termination of feeding in European corn borers occurs at the end of the final larval instar and signifies the onset of the wandering stage. Wandering was calculated as the number of days needed to reach the wandering stage after eclosion into the final larval instar. In non-diapause conditions wandering began on day 6 (3-3A) and on day 10 in diapause-programming conditions (3-3B) regardless of diapause genotype.

On the first day of the last larval instar, diapause-programmed larvae had accumulated larger lipid stores compared to their non-diapausing counterparts (t value=-2.73, Df=75.9, p-value=0.008) (3-5A). However, on day one there was no difference in fat stores between the two genotypes when reared within diapause-programming conditions nor did fat stores differ between genotypes in non-diapause conditions. Lean mass on the first day of the final larval instar was not different between the two genotypes regardless of rearing conditions (t-value=2.03, Df=5.9, p-value=0.089)(3-3A). Similarities in lean mass and lipid mass accumulation at the start of the final larval instar suggest that the two contrasting diapause genotypes exposed to the same conditions begin that final larval instar with the same amount of stored nutrition.

Once larvae reached the wandering stage, increases in lean mass accumulation and lipid stores were both clearly associated with diapause programming and diapause genotype. Larvae in diapause-programming conditions accumulated more lean mass and stored more fat than their counterparts in non-diapause conditions in both strains (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 both 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 in diapause-programmed larvae 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 were significantly lower when sampled 15 days after wandering in comparison to other sample days (t-value=-3.90, Df=111.4, p-value<0.000) (3-9C).