

**Resource Reservoir and the Decision to  
Diapause: The relationship between stored  
resources and diapause timing.**

**James T. Brown**

**MS Thesis Proposal**

**Advisor: Dr. Dan Hahn**

**Committee Member: Dr. Rob L. Meagher**

**Changing Climate:** Earth's climate is warming. According to the National Oceanic and Atmospheric Administration, 2016 was the warmest year on the record with global surface temperatures and North American land surface temperatures averaging 0.94 °C and 1.86 °C above the 20<sup>th</sup> century averages, respectively (NOAA National Centers for Environmental Information 2017). Conservative projections of future temperatures estimate at least a 1.5 °C increase in global surface temperature by the end of the 21<sup>st</sup> century with temperatures continuing to increase thereafter (DeLucia et al. 2008, Stocker et al. 2015). Seasonal temperature averages in the United States during 2016 echoed this upward trend for all four seasons surpassing all previously recorded temperature averages (NOAA National Centers for Environmental Information 2017). Warmer temperatures will effectively increase the duration of the warm growing season as fall, winter, and spring temperatures increase (Bradshaw and Holzapfel 2006, Hahn and Denlinger 2011, Scriber 2014). Generally, ectotherm metabolic rates correspond to the environmental temperatures they experience. Higher temperatures increase metabolic rates and lower temperatures reduce metabolic rates. Increased growing season temperatures for ectothermic insects could increase their metabolic rates, speed up their growth, and possibly shorten development time to reproductive maturity. Mature adults that occur earlier in the growing season could increase the number of generations each year (Bale et al. 2002, Bradshaw and Holzapfel 2006, Hahn and Denlinger 2011, Scriber 2014). For insect pests, warmer temperatures introduce the possibility of larger pest populations causing more damage to economically important crops. Managing these indirect effects of climate change will require an integrated approach, and likely increased use of costly chemical insecticides.

Crop losses due to insect pest insect damage here in the United States from 1945 to 2000 have nearly doubled from 7% to 13%, while insecticide use has increased 10-fold (Pimentel and Burgess 2014). Insecticide use can manage insect pest populations, but even with careful monitoring and systematic application regimens, pest insects can significantly reduce crop yields. Under current climate conditions and pest pressure, yield reductions in chemically managed, pre-harvest crops due to arthropods is estimated to be between 13%-16% annually (Oerke 2006). As warmer temperatures begin earlier in the year and end later, larger pest insect populations could lead to lower crop yields and the cost to manage these potentially larger and earlier occurring pest populations using chemical insecticides is likely to increase. Lower crop yields due to increased pest damage will endanger access to safe nutrient-rich foods for growing populations around the world. Here in the United States, the population is predicted to exceed 450 million by the year 2100 and this population increase will require sustained or even increased crop yields (Melorose et al. 2015). Investigating the responses of pest insect populations to increasing temperature is an opportunity to understand and predict how climate change could affect these pests. The results of such an investigation could be used to mitigate their damage and ensure the security of our nation's food as populations increase.

**Responses to Climate Change:** Animal performance is influenced by the thermal conditions they experience in their environments and increased temperatures could affect animals either positively or negatively (Huey and Stevenson 1979, Chown 2007). As climate changes, insects whose populations are impacted negatively by those changes can be colloquially termed

“losers” and those impacted positively can be termed “winners”. The direct and indirect interactions between temperature and the resulting winners could lead to expanded geographic ranges, increased population size, or increased temperature tolerance (Hughes 2000, Williams et al. 2008). An insect’s body temperature directly affects its performance, and the effect of body temperature on performance can be described using a thermal performance curve (Huey and Stevenson 1979). At the peak of this curve is an insect’s thermal optimum, this is the temperature where performance is maximized. The range of temperatures where the performance of an insect is half of the thermal optimum represents the thermal breadth. Finally, the range of temperatures within which any performance is permitted is an insect’s thermal tolerance range. Temperatures at the edge of an insects thermal tolerance are termed the critical thermal maximum and critical thermal minimum, respectively (Bale et al. 2002, Huey et al. 2012, Sinclair et al. 2016).

Warmer days arriving earlier in the spring and ending later in the fall will extend the duration of the warm growing season in temperate regions. In effect, the seasonal temperatures experienced in northern latitudes will resemble the seasonal temperatures of the adjacent southern latitudes, increasing the geographic distribution of warmer environments (Parmesan et al. 1999, Breed et al. 2012). Warmer temperatures could shrink the southern distribution of losing populations, reducing their population size. Insects that are unable to shift their geographic range of their population or unable to tolerate increasing temperatures in their current environment could be losers.

Winners could experience a net increase in both population size and geographical range

with more individuals spread across more geography. Winners could also experience a northern shift of their entire geographical range with no change in population size. In Europe, changes in ranges have been observed in 35 species of non-migratory butterfly species. Of these butterflies, 63% were observed to have a distribution shift northward and 3% were observed to have a distribution shift southward (Parmesan et al. 1999). As favorable thermal conditions for winning insects shift farther north and warmer days increase in frequency and duration, the spatial distribution of winning insects could track those favorable temperatures.

Warmer temperatures for losers could directly reduce their performance by exceeding their thermal breadth. Continued temperature increases for these losing insects could exceed their thermal maximum and eventually cause mortality. Winners in contrast could have wider thermal breadths and tolerate warmer temperatures. Winners, whose thermal environment is currently below their thermal optimum, could experience increased performance as temperatures increase towards their thermal optimum. In a review of the effects of thermal conditions on population fitness (with fitness defined as the intrinsic population growth of r-strategy insects), Deutsch et al. (2008) tracked and compared population size between 38 representative insect species from temperate and tropical latitudes. For those representative species across temperate latitudes, the thermal breadth of these insects tended to be wider and the thermal conditions experienced in these locations, on average, tended to be further away from their critical thermal maximum compared to the representative taxa from tropical latitudes (Deutsch et al. 2008). In the tropics, environmental temperatures vary little relative to temperatures in temperate regions and insects in tropical regions experience temperatures

that tend to be closer to their optimum temperature relative to temperate insects whose environment tends to be cooler than optimum. This suggests that tropical insects already live near their thermal limits and thus could quickly become losers as climate warms.

Seasonal changes in temperature are cyclic and correspondingly can delimit the availability of resources, like host plants for phytophagous insects. For plants and animals alike, temperature has a strong influence on their growth and performance, but daily temperatures can fluctuate from year to year. The ability to reliably predict seasonal changes is probably one of the most important challenges all organisms encounter.

To prepare for seasonal changes in temperature, many plants and animals synchronize their development using other environmental cues that consistently cycle with seasonal temperature changes. In the temperate regions farther from the equator, photoperiod consistently changes incrementally by latitude and season (Hut et al. 2013). During the summer, photoperiod is long and increases as latitude increases; while in the winter, photoperiod is short and decreases as latitude increases. Insects in temperate regions use the consistent, incremental changes in photoperiod at specific latitudes to synchronize their life histories with the availability of resources in their environment. With growing seasons beginning earlier and ending later each year as climate changes, a hypothetical photoperiod of 13 hours that previously indicated the average beginning of the growing season could, as temperatures increase, indicate on average the second week of the growing season instead. Warmer seasonal temperatures will uncouple photoperiod from seasonal changes in temperature and resource availability. Insects that depend on photoperiod to make life history

decisions, but cannot adjust to the warmer temperatures approximated by photoperiod, could lose.

Photoperiod, like temperature, is an important environmental cue that insects use to make life history decisions. While warming northern latitudes do offer climate change winners the opportunity to shift their population distributions and ranges. Those insects that experience range shifts will be exposed to environmental cues, like photoperiod, that are intrinsic to these northern latitudes. Winners could be pre-adjusted or could adjust to these shifted cues in the environments they relocate into through phenotypic plasticity or evolutionary adaptation. Phenotypic plasticity is defined as the capacity of a single genotype to express multiple, different phenotypes as a function of the environmental conditions that the genotype encounters (Agrawal 2001). Evolutionary adaptations are genetic changes that occur within populations due to selection (Lee 2002). Failure to adjust to the photoperiods of these warmer northern latitudes could negatively impact the timing of life history events for those shifted populations, turning winners into losers.

**Adjusting through Dormancy:** To ensure their survival, organisms must monitor both their internal condition as well as the external environment, and respond to changes in those environments as they occur. Insects must actively work to avoid conditions that become too stressful and take advantage of conditions that are favorable. Environmental stress that occurs over a relatively short period of time can be categorized as acute stress, while stress that occurs over a relatively prolonged period can be considered chronically stressful. Stress in an insect's

natural environment could be any condition that, if encountered, impacts growth, reproduction, or survival. Common environmental stresses for insects include extreme temperatures, ice, desiccation, and reductions in the availability of food. In general, dormancy is a state of metabolic and developmental suppression many insects use to mitigate the effects of both acute and chronic seasonal stress they encounter in their environment (Košťál 2006). As temperatures rise, wintering insects could express phenotypic plasticity or experience evolutionary adaptations in their dormancy strategy to adjust to the shifting landscape of seasonally stressful environmental conditions.

As insects monitor their environment and perceive acute environmental stress, some use quiescence to quickly respond to these relatively short-term conditions. Quiescence is a transient state of reduced activity that insects can use to temporarily protect themselves from environmental stress (Košťál 2006). Once the stress is relieved (provided the exposure was not too extensive), quiescence is reversed and the insect's activity can resume after some period of recovery. Seasonal temperature change is a common long-term stress that insects encounter in their environment. To avoid or mitigate the consequences of predictable seasonal environmental stress, many insects use diapause. For most temperate insects, maintaining a suitable metabolic rate for continued development becomes challenging when temperatures fall too low. Further, as resource availability declines, they struggle to acquire enough energy to fuel metabolism, growth, and development. Diapause is one way that insects can protect themselves from these predictable and chronic seasonal stresses. However, unlike quiescence, diapause is generally induced well before their environment degrades and becomes stressful.



Diapause is a genetically regulated, environmentally influenced alternative developmental trajectory that is usually marked by metabolic suppression and arrested development in a specific life stage (Košťál 2006). By monitoring environmentally consistent cues that cycle with seasonality, insects can reliably predict, prepare for, and protect themselves from seasonal changes in temperature by inducing diapause.

Within a single insect species, the environmental cues that stimulate diapause, the life stages sensitive to those cues, and the resulting diapause phenotype are typically consistent (Bale and Hayward 2010). The traits that mark diapause are largely genetically determined and highly heritable. The developmental stage when diapause occurs can vary from species to species or can even vary among populations within a species. Variation in diapause life stage aside, the diapause developmental trajectory always has three sequential stages: pre-diapause, diapause, and post-diapause. Before diapause can be induced, an individual must reach a genetically determined sensitive period. Sensitive insects can perceive the environmental cue or cues that induce diapause, and during this period they are physiologically competent to respond to that cue or cues. During pre-diapause, the sensitive stage perceives the necessary environmental cue or cues, diapause is induced, and there is a shift away from continuous development and towards the diapause developmental trajectory.

During diapause, insects must meet their metabolic energy requirements. However, most insects do not feed during diapause. Generally, diapause is induced before an insect experiences seasonal changes in their environment. Preemptive induction of diapause provides insects the opportunity to accumulate and store resources needed to survive diapause before

seasons change (Košťál 2006). In preparation for diapause, many insects accumulate and store resources in the form of lipids, proteins, and carbohydrates. Because most insects do not feed during diapause, it is imperative that insects accumulate enough resources to meet the energetic demands of the long diapause period. Furthermore, after diapause ends insects must have enough resources remaining to meet the anabolic requirements for development, metamorphosis, repair, and post-diapause activities like reproduction (Hahn and Denlinger 2007, Sinclair 2015). Following the successful completion of the diapause preparatory phase, insects enter diapause, progressing through three distinct stages: initiation, maintenance, and termination.

Diapause initiation is generally marked by the suspension of continuous development and a reduction in metabolic activity (Tauber and Tauber 1981, Košťál 2006, Hahn and Denlinger 2007, Sinclair 2015). During diapause maintenance, the endogenous mechanisms that support the diapause phenotype persist and diapause continues (Košťál 2006). Diapause termination is marked by the relief of those endogenous factors that initiate and maintain diapause, allowing development to resume under permissive conditions (Košťál 2006). After diapause is terminated, the potential to resume development exists. However, many insects do not immediately resume development. Instead, under non-permissive conditions, post-diapause insects remain quiescent and their development is arrested by exogenous environmental factors like low temperatures. When the exogenous factors permissive to growth become available development can resume (Košťál 2006).

Diapause is an alternative life history trajectory that requires an insect to monitor

environmental cues, halt development, and suppress metabolic activity. The timing of diapause is crucial because developmental arrest and metabolic suppression can produce profound behavioral and physiological changes. If an insect enters diapause too late they could expose themselves to stressful environmental conditions and if diapause ends too soon the environment may not be suitable for that insect's growth and development, or mates may not be available for reproduction. The mechanisms controlling diapause timing as an important life history decision are endogenously regulated and typically genetically heritable.

In temperate regions, warm temperatures persist in the spring and summer. During the warm seasons, insects utilize available food and water to grow, develop, and reproduce. As temperatures decline in the fall and winter, resource availability declines. For insects in temperate regions, low temperatures can greatly reduce metabolic activity making continued activity challenging or even impossible. Consequently, low metabolic activity reduces growth and can eventually cause mortality. Insects in temperate regions generally predict the seasonal change using photoperiod alone or in concert with other environmental cues, like temperature or host-plant quality, to induce diapause and avoid the stress of prolonged seasonal stress in their environments.

As climate changes and average seasonal temperatures increase, the duration of the warm growing season is expected to increase. With growing seasons beginning earlier and ending later, some of the seasonal cues insects use to predict changes in their environment, like photoperiod, will not change. In time, the predictions of those unchanged environmental cues will become decoupled from actual seasonal changes as growing seasons become longer and

winter shrinks. Environmental cues that previously signaled the end of the growing season will underestimate the end of the longer growing season. Those insects that adjust to these underestimated predictions to resynchronize their lifecycles with the growing season, either by evolutionary adaptations or phenotypic plasticity in their response to these shifting environmental cues, could win as climate changes.

The pitcher plant mosquito provides one example of how insects could adjust to longer and warmer growing seasons through evolutionary adaptation. Bradshaw and Holzapfel (2001) showed that populations of the pitcher plant mosquito, *Wyeomyia smithii*, have shifted their critical photoperiods for diapause induction to extend their growing season, consistent with predictions for climate change. Critical photoperiod is the number of light hours required to induce diapause in 50% of a population. In *W. smithii* the critical photoperiod for diapause induction is highly heritable. As larvae, pitcher plant mosquitos grow and develop in the in the water-filled leaves of pitcher plants. These mosquitos inhabit temperate regions as far south as the Gulf of Mexico and as far north as northern Canada. Across this wide latitudinal range, pitcher plant mosquitoes experience their longest growing seasons at the southern end of their range and increasingly shorter growing seasons at more northern latitudes. At the end of the warm growing season, photoperiod gets shorter. Once photoperiod drops below a genetically determined number of light hours, larvae perceive that cue enter the larval diapause developmental trajectory.

Bradshaw and Holzapfel (2001) sampled several populations of *W. smithii* larvae from latitudes between Florida and Canada in the years 1972, 1988, 1993, and 1996 and reared them

in a common-garden laboratory setting under strict environmental control. Populations collected in 1972 and 1996 were exposed to a range of different photoperiods to determine their critical photoperiod (Bradshaw and Holzapfel 2001). In 1972, the critical photoperiod of larvae populations collected at 50 °N, averaged 15.79 hours while the critical photoperiod of larvae populations collected in 1996 at the same latitude averaged 15.19 hours.

Because of the rigor with which these experiments were conducted and the highly heritable nature of critical photoperiod within this species, these results suggest the populations collected in 1996 have evolved and are now genetically different than populations collected in 1972. Northern pitcher plant mosquitoes, on average, are delaying diapause by approximately 9 days and this shift correlates with the average increase in the number of warmer days experienced in this region (Bale and Hayward 2010). Delayed diapause initiation could be evolutionarily adaptive. For pitcher plant mosquitoes, warmer temperatures are indirectly responsible for the increased availability of environmental resources these mosquitoes need to grow and develop. The mosquitoes that delay diapause initiation could access those resources and continue to grow, develop, and reproduce for an additional 9 days. For some insects, warmer seasonal temperatures and longer growing seasons will increase the duration of resource availability. Insects that can adjust to longer growing seasons without compromising the protection of diapause could be winners as climates change.

**Measuring the Descent into Diapause:** During diapause, temperatures are low and metabolic activity may be suppressed. However, insects can metabolize considerable quantities of

nutrients during this period. In preparation for diapause, some insects accumulate large quantities of lipids, amino acids, and/or carbohydrates. For some insects, the nutrients accumulated prior to diapause initiation must also be utilized for metamorphosis or to supplement a restricted diet once diapause is terminated. Lipids, specifically triglycerides, are the predominant source of metabolic energy used during diapause in most species (Arrese and Soulages 2010, Hahn and Denlinger 2011). Triglycerides can be accumulated directly from an insect's diet or synthesized in the fat body from amino acids or carbohydrate intermediates (Hahn and Denlinger 2007, Arrese and Soulages 2010). Amino acids are generally stored as multimeric hexamerin proteins. Hexamerins are specialized proteins that build up in the insect fat body or hemolymph prior to diapause (Burmester and Scheller 1999). These large protein complexes function as amino acid reservoirs. During diapause, as metabolic proteins accumulate damage or are destroyed, the amino acids in hexamerins could be mobilized and used to repair or replace damaged proteins (Burmester 1999, Hahn and Denlinger 2007). After diapause, hexamerin proteins could be catabolized and the constituent amino acids can be used to build exoskeleton, repair damaged proteins, and build new tissues during morphogenesis (Burmester 1999, Hahn and Denlinger 2007). Carbohydrates are polymerized and stored as glycogen in the fat body or as trehalose in the hemolymph (Hahn and Denlinger 2007, Arrese and Soulages 2010).

Preparations for prolonged low temperatures and the absence of environmental resources requires some insects to accumulate and store proportionally more lipids than carbohydrates or proteins to fuel their metabolism. For example, diapausing female *Culex pipiens* mosquitos

reared at 22 °C and under a 9-hour photoperiod accumulate significantly more lipid in preparation for diapause relative to their non-diapausing conspecifics reared at the same temperature and under a 14-hour photoperiod. These stored lipids are utilized as a source of energy during diapause (Mitchell and Briegel 1989). In other insects, diapause preparation has been shown to lead to an increase in hexamerin storage, as observed in the Colorado potato beetle, *Leptinotarsa decimlineata*. When Colorado potato beetles were laboratory reared under two different photoperiods, a 10-hour photoperiod to induce diapause and an 18-hour photoperiod to bypass diapause, diapause-programmed beetles had substantially higher transcript abundance of the hexamerin diapause protein 1 (De Kort and Koopmanschap 1994).

As climate changes, warm summers will begin earlier and end later followed by shorter and warmer winters. Increasing temperatures will generally increase metabolic activity in insects, including dormant insects, and increased metabolic activity will require more nutrients to fuel metabolism. Nutrients accumulated by insects in preparation for diapause at the end of the growing season, and used during diapause, could be affected by increased metabolism due to increased environmental temperatures. These changes could potentially affect both survival through diapause and/or post-diapause performance. In preparation for diapause, climate change losers could be unable to accumulate or store enough nutrients possibly resulting in an energy deficit at the beginning of diapause. During diapause, losers encountering increased temperatures could deplete their reservoirs of stored nutrients to meet their increased metabolic demands before diapause ends and not survive the winter. Insects able to accumulate more nutrients during pre-diapause or properly allocate stored resources to

support their increased metabolism during diapause could be winners as climate changes. Quantifying the metabolic demand for nutrient storage in preparation for diapause as a function of diapause length could provide a way to predict climate change winners and losers as growing seasons increase in duration. *Ostrinia nubilalis* (European corn borer) is a naturally occurring insect pest with sympatric populations that are genetically distinct and express diapause phenology that differs in length. These traits make ECB a suitable model to understand the association between diapause timing and nutrient demands.

European corn borer (ECB) is a phytophagous lepidopteran that occurs in most states east of the Rocky Mountains from Canada to the Gulf of Mexico (Beck and Apple 1961, Capinera 2000, Bohnenblust and Tooker 2010). ECB populations are categorized into strains characterized by voltinism and pheromone composition. Voltinism represents the annual number of generations produced by a population before entering diapause (Dopman et al. 2005). Across its geographical distribution, ECB populations separate clinally with voltinism increasing from univoltine at the northern edge to bivoltine and subsequently multivoltine populations as latitude decreases (Beck and Apple 1961). ECB strains are further characterized by the composition of their sex pheromone. Sex pheromone biosynthesis in ECB females involves the  $\beta$ -oxidation of palmitic acid into (E)-11-tetradecenoyl and (Z)-11-tetradecenoyl precursors which can be reduced into their corresponding fatty alcohols then acylated into a pheromone molecule (Lassance et al. 2010). The specific ratio of precursor molecules converted into pheromone differs between two naturally segregating z-chromosome genetic variants (Lassance et al. 2010). The gene responsible for pheromone synthesis has two different alleles.



The higher concentration of (Z)-11-tetradecenyl acetate in the Z strain sex pheromone blend is due to the affinity of (Z)-11-tetradecenoyl precursors to the fatty acid reductase enzyme produced from the *pgFAR-Z* allele (Lassance et al. 2010). Alternatively, the high concentration of (E)-11-tetradecenyl acetate characteristic of the E strain is due to the increased affinity of (E)-11-tetradecenoyl precursors to the fatty acid reductase produced from the *pgFAR-E* allele (Lassance et al. 2010).

The onset of diapause in the species *Ostrinia nubilalis* is determined by the interaction between photoperiod and temperature. However, differences in diapause length between the bivoltine and univoltine strains are associated with differences at a genomic factor located on the Z sex chromosome (Dopman et al. 2005). During the larval stage, ECB predicts seasonal changes by monitoring changes in photoperiod during the warm growing season. As the growing season comes to an end, photoperiod decreases. Short days perceived by ECB during the 5<sup>th</sup> instar induce a post-feeding larval diapause. The *Pdd* region of the Z-chromosome is a major factor associated with diapause length and is partially responsible for determining voltinism during the growing season. The univoltine-Z (UZ) and bivoltine-E (BE) genotypes express longer and shorter diapause phenology respectively, as well as differences in their pheromone blend. Univoltine-Z (UZ) strain larvae enter diapause earlier in the fall and exit diapause later in the spring compared to the BE genotype. Under controlled laboratory conditions, the unique response of each strain can be reproducibly observed.

I hypothesize that the quantity of triglycerides stored by European corn borers, in preparation for the additional stress of diapause, will be associated with differences in diapause

length between univoltine-Z and bivoltine-E *Ostrinia nubilalis* strains. Specifically, I predict that the UZ genotype of ECB preparing for a longer period of diapause will store relatively more triglycerides than the BE genotype, which will have a shorter diapause. In support of the stated hypothesis, I predict that non-diapausing larvae will store fewer triglycerides than diapausing larvae within each strain because they do not have the added metabolic cost of diapause. The goal of this study is to quantify and identify accumulated triglycerides in diapausing and non-diapausing ECB larvae of each strain and determine the degree to which accumulated triglyceride can be associated with diapause length.

Hypothetically, longer and warmer growing seasons could extend the duration of suitable environments and available resources for insects. Longer and warmer growing seasons have the potential to increase insect feeding, mating, and voltinism. Climate change will affect insect populations, and how insects respond to climate change will determine which insects are losers and which are winners, ECB is no exception. European corn borer is a major agricultural pest here in the United States, accounting for up to \$2 billion dollars in costs associated with managing these pests (Hyde et al. 1999). Investigating the energy requirements of diapause could expose mechanisms that regulate the timing of this tenuous life history decision. Developing strategies to manipulate the mechanisms regulating the progression of ECB through diapause could be valuable. Eventually, perturbing the European corn borer larvae's ability to survive diapause by affecting how it accumulates and stores resources in preparation for diapause could be used as an added layer of pest management. Until then, the link between seasonal temperatures and global food security will become more tenuous and finding a

making a comprehensive approach to dealing with the response of pest insects to climate change is imperative.

## **PROPOSED METHODOLOGY**

**Origin and Husbandry of European Corn Corer:** The Univoltine-Z (UZ) and Bivoltine-E (BE) strains of ECB that will be used in this experiment were collected by members of Dr. Eric Dopman's laboratory at Tufts University. Strain identity was determined genotypically using the *pgFAR* autosomal gene, this gene codes an important enzyme involved in determining the female sex-pheromone blend, and is partly responsible for the strain differences (Lassance et al. 2010). Both strains were collected as a mixture of larvae, pupae, and adults from New York state prior to 2015 (Wadsworth et al. 2015). For the duration of the experiment, each strain will be continuously mass reared at 26 °C under a 16L:8D photoperiod to promote continuous development. To test for differences in stored triglycerides between the diapause and non-diapause larvae, newly hatched larvae from each strain will be reared at 23 °C under a 12L:12D photoperiod to induce diapause or 16L:8D photoperiod to promote continuous development. Those larvae treated under diapause-inducing conditions from the UZ and BE strains will be labeled UZ12 and BE12 respectively and those treated under diapause avoiding conditions will be labeled UZ16 and BE16 respectively.

**Sampling Wandering Larvae.** European corn borer eggs intended for non-diapause and diapause treatment from the UZ and BE strains will be held at 23 °C and 65% relative humidity

and under a 16-hour photoperiod until they hatch. Upon hatching these larvae will be provided artificial ECB diet ad libitum, purchased from Frontier Agricultural Sciences. After hatching, non-diapause treatment larvae will be reared under a 16L:8D photoperiod, while diapause treatment larvae will be reared under a 12L:12D photoperiod. The photoperiod regime experienced by each treatment will be held constant throughout the duration of the experiment. At the beginning of the fifth instar, larvae will be separated into individual arenas within 32-well bioassay trays purchased from Frontier Agricultural Sciences. Once larvae reach the end of the fifth instar, they will be assayed to determine if they have entered the wandering phase. At the beginning of the wandering phase, larvae discontinue feeding, empty the contents of their gut, and after clearing their gut larvae no longer produce frass. The wandering phase is a necessary step all ECB larvae undergo in preparation for either diapause or larval-pupal metamorphosis (Gelman and Hayes 1982). Wandering larvae terminate feeding and frass production ends. The lack of frass production will be used to diagnose putative wandering larvae. To diagnose late fifth instar larvae as wandering, larvae will be removed from their individual arenas and held in a clean, empty petri dish and monitored for 4 hours. Larvae whose gut is not clear will produce frass and will be placed back into their arenas and larvae that do not produce frass will be characterized as wanderers. Samples of 60 larvae will be collected from each photoperiod regime (diapause and non-diapause) consisting of 30 larvae from each strain (UZ and BE). Each collected larva will be accessioned and tracked for the duration of the experiment. Lipid extractions from each individual sampled larva will be analyzed for triglyceride quantity and identity.

**Lipid Extraction, Separation, and Quantification:** The total lipid content from each larva will be extracted and quantified individually. First, each frozen larval sample will be lyophilized under vacuum at -80 °C. When the larval weight varies by less than 1% over a 24-hour period, larval dry mass will be recorded. Lipids from each larvae will be separated from the larval tissues using a slightly modified Folch method (Gossert et al. 2011). This method takes advantage of the polarity and density differences between hexanes and methanol that allow each solvent to selectively solubilize molecules of similar polarity and to produce distinct layers when mixed together. When a larval sample is solubilized in this solvent mixture, the less polar lipids will be captured in the less polar hexanes layer. The hexanes layer is then removed using a Pasteur pipette and the total amount of lipids extracted from the sample can be quantified gravimetrically. The extracted lipids will contain a mixture of different lipid classes including triglycerides. Separating and quantifying triglycerides from the total extracted lipids will be accomplished using ultra performance liquid chromatography (UPLC) coupled with an evaporative light scattering detector (ELSD) and an Agilent Zorbax Eclipse XBD C18 column. UPLC takes advantage of the physical properties of lipid molecules to adsorb to stationary phase that lines a column and interact with a mobile phase. The column's stationary phase is a matrix of proprietary silica gel backbone bound with dense monolayer of dimethyl-n-octadecylsilane (C18) moieties. The mobile phase includes two solutions; mobile phase A is a 0.1% acetic acid in methanol mixture and mobile phase B is 40% hexanes in 2-propanol. Samples are injected onto the column and the contained lipids adsorb to the C18 silica matrix.

Over time, the concentration of the mobile phase shifts from 100% A to 100% B. As the gradient changes, different classes of lipid molecules with successively lower polarities will desorb from the column flow into the ELSD where they are nebulized, the mobile phase is evaporated, and the lipids in the sample scatter light emitted by the detector. As the amount of light scattered by the lipids is detected, a response is generated that corresponds to the amount of lipids present. The response output of the ELSD can then be quantified by comparing it to the response output of a standard concentration of triglycerides. Tristerin and tripalmitin acid (purchased from Sigma Millipore) and triheptadecanoin (purchased from VWR) will be used to prepare the standardized mixture of triglycerides.

**Lipid Identification:** To identify the fatty acid components of the triglycerides quantified by UPLC-ELSD, the triglycerides will be esterified into fatty acid methyl esters (FAMES). Triglycerides will be methylated via base-catalyzed esterification with an acid catalyzed work-up (Christie 1993, Liu 1994). Extracted lipids will be mixed in a solution of 10M methanolic potassium hydroxide at 55 °C for thirty minutes in a capped vial. The heated sample will be vortexed for two minutes and then cooled on ice for five minutes. While still on ice, 12M sulfuric acid will be added to neutralize the KOH and terminate the reaction. After the reaction is terminated 3 mL of hexanes will be added to the reaction vial to solubilize the FAMES. The hexanes layer containing the esterified lipids, will then be removed using a Pasteur pipette and any water species formed by the esterification procedure will be removed using sodium sulfate. Identification of the methyl-ester species will be accomplished using gas chromatography (GC)

coupled with a flame ionization detector (FID). GC-FID separates each FAME by taking advantage of the specific interactions between different FAMES and the packing material in a DB-WAX capillary column. The FAMES in the sample adsorb onto the column and inert gas flows through the column. Over time, the column temperature increases, the compounds volatilize, and the FAME molecules desorb from the column based on their interaction with the column packing material and the inert gas carries them to the detector. At the detector, retention time is recorded as each FAME molecule is ionized by flame and the intensity of ionization is recorded as a response output. FAMES will be identified in comparison to a standard purchased from Sigma-Millipore (37 Component FAME Mix, CRM47885).

**Data Analysis:** Using the techniques mentioned above, we predict that larvae exposed to a 12L:12D photoperiod will accumulate more triglycerides in preparation for diapause compared to continuously developing larvae exposed to a 16L:8D photoperiod. Additionally, the longer diapausing univoltine-Z strain larvae should accumulate more triglycerides than the shorter diapausing bivoltine-E larvae. Prior to the compilation of this proposal, preliminary investigations to quantify triglyceride accumulation have already yielded results in line with our predictions. The average of the total lipid accumulated was compared between diapausing and non-diapausing UZ larvae. These data suggest there is a significant difference between the total lipid content of diapausing and non-diapausing UZ larvae ( $Df = 1$ ,  $p = 1.06 \times 10^{-7}$ ). One interpretation of these data could be that as these larvae perceive a 12 hour photoperiod they experience physiological changes that alter the accumulation and storage of lipids in

preparation for diapause. To support this initial result, replication of these experiments will need to be conducted. These and future measurements of triglyceride from each of the four treatments will be expressed as concentrations. Total triglyceride concentration will be determined as the total sum of the triglyceride peak areas in relation to the peak area of an external standard of known triglycerides at known concentrations. A multivariate analysis of accumulated lipids will be used to explore the significance of interactions between measured parameters. Measured parameters include: larval wet mass, lean mass, and dry mass, total lipid mass, temperature, analysis block, and photoperiod. To explore the effect of strain and photoperiod on lipid accumulation, a mixed linear model will be used to model significant factors and their interactions. Within the mixed model ECB strain, photoperiod, and cohort will be designated as fixed factors, lean mass will be designated as a covariate, and each analysis block will be defined as a random factor. Investigating these interactions could determine if there are interesting patterns and could help explain the variation we see in the lab and nature.



## REFERENCES:

- Agrawal, A. A. 2001.** Phenotypic Plasticity in the Interactions and Evolution of Species. *Science* (80-. ). 294: 321–326.
- Arrese, E. L., and J. L. Soulages. 2010.** Insect Fat Body: Energy, Metabolism, and Regulation. *Annu. Rev. Entomol.* 55: 207–225.
- Bale, J. S., and S. A. L. Hayward. 2010.** Insect overwintering in a changing climate. *J. Exp. Biol.* 213: 980–994.
- Bale, J. S., G. J. Masters, I. D. Hodkinson, C. Awmack, T. M. Bezemer, V. K. Brown, J. Butterfield, A. Buse, J. C. Coulson, J. Farrar, J. E. G. Good, R. Harrington, S. Hartley, T. H. Jones, R. L. Lindroth, M. C. Press, I. Symrnioudis, A. D. Watt, and J. B. Whittaker. 2002.** Herbivory in global climate change research: Direct effects of rising temperature on insect herbivores. *Glob. Chang. Biol.* 8: 1–16.
- Beck, S. D., and J. W. Apple. 1961.** Effects of Temperature and Photoperiod on Voltinism of Geographical Populations of the f, *Pyrausta nubilalis*. *J. Econ. Entomol.* 54: 550–558.
- Bohnenblust, E., and J. Tooker. 2010.** European Corn Borer in Field Corn.
- Bradshaw, W. E., and C. M. Holzapfel. 2001.** Genetic shift in photoperiodic response correlated with global warming. *Proc. Natl. Acad. Sci. U. S. A.* 98: 14509–14511.
- Bradshaw, W. E., and C. M. Holzapfel. 2006.** Evolutionary response to rapid climate change. *Science* (80-. ). 312: 1477–1478.
- Breed, G. A., S. Stichter, and E. E. Crone. 2012.** Climate-driven changes in northeastern US butterfly communities. *Nat. Clim. Chang.* 3: 142–145.
- Burmester, T. 1999.** Evolution and function of the insect hexamerins.pdf. *Eur. J. Entomol.* 96: 213–225.
- Burmester, T., and K. Scheller. 1999.** Ligands and receptors: Common theme in insect storage protein transport. *Naturwissenschaften.* 86: 468–474.
- Capinera, J. L. (Entomology and N. D. 2000.** European corn borer scientific name : *Ostrinia nubilalis* ( Hübner ) ( Insecta : Lepidoptera : Pyralidae ).
- Chown, S. L. 2007.** Physiological diversity in insects: ecology and evolutionary contexts. *Adv. In Insect Phys.* 33: 50–152.
- Christie, W. W. 1993.** Preparation of Ester Derivatives of Fatty Acids for Chromatographic Analysis. *Adv. Lipid Methodol.* 69–111.
- DeLucia, E. H., C. L. Casteel, P. D. Nabity, and B. F. O'Neill. 2008.** Insects take a bigger bite out of plants in a warmer, higher carbon dioxide world. *Proc. Natl. Acad. Sci.* 105: 1781–1782.
- Deutsch, C. A., J. J. Tewksbury, R. B. Huey, K. S. Sheldon, C. K. Ghalambor, D. C. Haak, and P. R. Martin. 2008.** Impacts of climate warming on terrestrial ectotherms across latitude. *Proc. Natl. Acad. Sci.* 105: 6668–6672.
- Dopman, E. B., L. Perez, S. M. Bogdanowicz, and R. G. Harrison. 2005.** Consequences of reproductive barriers for genealogical discordance in the European corn borer. *Proc. Natl. Acad. Sci.* 102: 14706–14711.
- Gelman, D. B., and D. K. Hayes. 1982.** Methods and Markers for Synchronizing Maturation of Fifth-Stage

- Larvae and Pupae of the European Corn Borer, *Ostrinia nubilalis*. *Ann. Entomol. Soc.* 75: 485–493.
- Gossert, A. D., A. Hinniger, S. Gutmann, W. Jahnke, A. Strauss, and C. Fernández. 2011.** A simple protocol for amino acid type selective isotope labeling in insect cells with improved yields and high reproducibility. *J. Biomol. NMR*.
- Hahn, D. A., and D. L. Denlinger. 2007.** Meeting the energetic demands of insect diapause: Nutrient storage and utilization. *J. Insect Physiol.* 53: 760–773.
- Hahn, D. A., and D. L. Denlinger. 2011.** Energetics of Insect Diapause. *Annu. Rev. Entomol.* 56: 103–121.
- Huey, R. B., M. R. Kearney, A. Krockenberger, J. A. M. Holtum, M. Jess, and S. E. Williams. 2012.** Predicting organismal vulnerability to climate warming: roles of behaviour, physiology and adaptation. *Philos. Trans. R. Soc. B Biol. Sci.* 367: 1665–1679.
- Huey, R. B., and R. D. Stevenson. 1979.** Integrating thermal physiology and ecology of ectotherms: A discussion of approaches. *Integr. Comp. Biol.* 19: 357–366.
- Hughes, L. 2000.** Biological consequences of global warming: Is the signal already apparent? *Trends Ecol. Evol.* 15: 56–61.
- Hut, R. A., S. Paolucci, R. Dor, C. P. Kyriacou, and S. Daan. 2013.** Latitudinal clines: an evolutionary view on biological rhythms. *Proc. R. Soc. B Biol. Sci.* 280: 20130433–20130433.
- Hyde, J., M. A. Martin, P. V Preckel, and C. R. Edwards. 1999.** The economics of Bt corn: valuing protection from the European Corn Borer. *Rev. Agric. Econ.* 21: 442–454.
- IPCC. 2013.** Summary for Policymakers. In: *Climate Change 2013: The Physical Science Basis. Contribution of Working Group I to the Fifth Assessment Report of the Intergovernmental Panel on Climate Change*, CEUR Workshop Proc. Cambridge University Press, Cambridge.
- De Kort, C. A. D., and A. B. Koopmanschap. 1994.** Pergamon 0022-1910(93)EOO24-V Nucleotide and Deduced Amino Acid Sequence of a cDNA Clone Encoding Diapause Protein 1, an Arylphorin-type Storage Hexamer of the Colorado Potato Beetle. *J. Insect Physiol.* 40: 527–535.
- Košťál, V. 2006.** Eco-physiological phases of insect diapause. *J. Insect Physiol.* 52: 113–127.
- Lassance, J.-M., A. T. Groot, M. A. Liénard, B. Antony, C. Borgwardt, F. Andersson, E. Hedenström, D. G. Heckel, and C. Löfstedt. 2010.** Allelic variation in a fatty-acyl reductase gene causes divergence in moth sex pheromones. *Nature.* 466: 486–489.
- Lee, C. E. 2002.** Evolutionary genetics of invasive species. *Trends Ecol. Evol.* 17: 386–391.
- Liu, K. S. 1994.** Preparation of fatty acid methyl esters for gas-chromatographic analysis of lipids in biological materials. *J. Am. Oil Chem. Soc.* 71: 1179–1187.
- Melrose, J., R. Perroy, and S. Careas. 2015.** World population prospects ( No. ESA/P/WP.241), United Nations.
- Mitchell, C. J., and H. Briegel. 1989.** Inability of diapausing *Culex pipiens* (Diptera: Culicidae) to use blood for producing lipid reserves for overwinter survival. *J. Med. Entomol.* 26: 318–26.
- NOAA National Centers for Environmental Information. 2017.** State of the Climate: Global Climate Report for Annual 2016. January. (<https://www.ncdc.noaa.gov/sotc/global/201613>).
- Oerke, E.-C. 2006.** Crop losses to pests, p. 31. *In J. Agric. Sci.*
- Parmesan, C., N. Ryrholm, C. Stefanescu, J. K. Hill, C. D. Thomas, H. Descimon, B. Huntley, L. Kaila, J.**

- Kullberg, T. Tammaru, W. J. Tennent, J. a Thomas, and M. Warren. 1999.** Poleward shifts in geographical ranges of butterfly species associated with regional warming. *Nature*. 399: 579–583.
- Pimentel, D., and M. Burgess. 2014.** Environmental and economic costs of the application of pesticides primarily in the United States. *Integr. Pest Manag.* 3: 47–71.
- Scriber, J. M. 2014.** Climate-driven reshuffling of species and genes: Potential conservation roles for species translocations and recombinant hybrid genotypes, *Insects*.
- Sinclair, B. J. 2015.** Linking energetics and overwintering in temperate insects. *J. Therm. Biol.* 54: 5–11.
- Sinclair, B. J., K. E. Marshall, M. A. Sewell, D. L. Levesque, C. S. Willett, S. Slotsbo, Y. Dong, C. D. G. Harley, D. J. Marshall, B. S. Helmuth, and R. B. Huey. 2016.** Can we predict ectotherm responses to climate change using thermal performance curves and body temperatures? *Ecol. Lett.* 19: 1372–1385.
- Tauber, C. A., and M. J. Tauber. 1981.** Insect seasonal cycles: genetics and evolution ,~4195. *12*: 281–308.
- Wadsworth, C. B., X. Li, and E. B. Dopman. 2015.** A recombination suppressor contributes to ecological speciation in *OSTRINIA* moths. *Heredity (Edinb)*. 114: 593–600.
- Williams, S. E., L. P. Shoo, J. L. Isaac, A. A. Hoffmann, and G. Langham. 2008.** Towards an Integrated Framework for Assessing the Vulnerability of Species to Climate Change. *PLoS Biol.* 6: e325.