**Mechanisms Mediating the Descent into Diapause: The relationship between stored resources and diapause timing.**

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**MS Thesis Proposal**

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**Changing Climate:** Earth’s climate is warming. According to the National Oceanic and Atmospheric Administration, 2016 as the warmest year on the record with global surface temperatures and North American land surface temperature averages were 0.94°C and 1.86°C above the 20th century averages, respectively (NOAA National Centers for Environmental Information 2017). Additionally, conservative projections of future temperatures estimate at least a 1.5°C increase in global surface temperature by the end of the 21st century and 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 and average temperatures for spring, summer, fall, and winter all surpassed 20th-century temperature averages (NOAA National Centers for Environmental Information 2017). In temperate regions, annual seasonal temperatures can peak in the summer above 90°C and in the winter temperatures can decrease below freezing. As annual temperatures continue to increase, warmer days will begin earlier in the year and end later in the year, reducing the number of cool days in the spring and fall, effectively increasing the number of warmer days and the duration of summer (Bradshaw and Holzapfel 2006, Hahn and Denlinger 2011, Scriber 2014). For many organisms, warmer temperatures generally increase development and for these organisms, more frequent warmer days during the year could favor more development during these warmer seasons. As it relates to insects these longer, warmer seasons could provide more time for development that could be directed towards more resource gathering, mate finding, or reproduction possibly leading to increased populations (Bale et al. 2002, Bradshaw and Holzapfel 2006, Hahn and Denlinger 2011, Scriber 2014). For insect pests, managing the potentially damaging effects caused by larger insect pest populations will require an integrated approach and increased use of chemical insecticides. Insecticide use can manage insect pest populations, but even under strict application regimens insects can significantly reduce crop yields. Under current climate conditions, yield reductions in chemically managed, pre-harvest crops due to arthropods is estimated between 13%-16% annually (Culliney 2014). Crop loss due to insect pest insect damage here in the United States from 1945 and ending in 2000, has nearly doubled from 7% to 13% while insecticide use has increased 10-fold (Pimentel and Burgess 2005). 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. As temperatures continue to rise, lower crop yields, due to insect damage, will put into jeopardy access to safe nutrient rich foods for people in developed and developing countries around the world. Here in the United States, the citizen population is predicted to exceed 450 million by the year 2100 and this population increase will demand sustained or even increased crop yields (Melorose et al. 2015). Investigating the responses of pest insect populations to increases in temperature is an opportunity to better understand and predict how climate change could affect these pests and using those predictions mitigate their damaging effects and ensure the security of a nation’s food as populations increase.

**Responses to Climate Change:** Because the performance of all animals is influenced by the thermal conditions they experience in their environments, increased temperatures could affect animals either positively or negatively (Huey and Stevenson 1979, Chown and Terblanche 2006). In temperate regions, most herbivorous insects have evolved to grow during warmer seasons. As seasonal temperatures increase, the duration of the warm growing season will increase with warmer days that arrive earlier in the spring and end later into fall. In effect, warmer seasonal temperatures in northern latitudes will resemble the seasonal temperatures their adjacent southern latitudes increasing the geographic distribution of warmer environments (Parmesan et al. 1999, Breed et al. 2012). Those temperate insects that are sensitive to rising temperatures could exhibit some response to these higher temperatures. Insects impacted negatively will be “losers” and those impacted positively will be “winners”. Losing insects unable to tolerate warmer temperatures could experience reductions in timing of daily or seasonal periods of activity, reduced geographical distribution and reductions in population sizes. Alternatively, winning insects that tolerate these thermal changes and could experience increased daily and seasonal timing of activity, they could experience changes in their population distribution to track thermal ranges that permit their growth, or they could win by adapting to warmer temperatures through plasticity or genetic variation (Hughes 2000, Williams et al. 2008). Understnading the impact of warmer temperatures and how pest insects will respond to these temperature changes, could help predict and possibly mitigate the damaging effects winning pest insects could have on agricultural crops.

Generally, the range of temperatures that maximize the growth and performance of an animal, is defined as a thermal optimum, those temperatures that simply permit growth are considered the thermal breadth and those upper and lower temperatures that limit an animal’s survival (beyond which performance declines or is inhibited) are the critical thermal maximum and minimum, respectively (Huey and Stevenson 1979, Bale et al. 2002). As warmer days begin earlier in the year and last longer, losing insects could be unable to tolerate any increases in temperature. Specifically, the range of temperatures that make up a losing insect’s thermal breadth could be narrow or the thermal conditions experienced by losing insects could already be near their critical thermal maximum. For these insects, rising daily and seasonal temperatures reduce their activity and exceed their critical thermal maximum earlier in the day and earlier in the season, inhibiting their growth and eventually leading to mortality. Winners, in contrast, could be those insects with a wider thermal breadth or those insects whose current thermal environment is closer to their critical thermal minimum. For these insects, rising temperatures could be tolerated and could even lead to increased daily and seasonal periods of activity. For some insects warmer temperatures could even lead to maximized growth as temperatures increase within their thermal optimum range. In a review of species fitness (where fitness is defined as the intrinsic population growth of r-strategy insects) and average thermal conditions, population size is tracked and compared between 38 representative insect species from temperate and tropical latitudes (Deutsch et al. 2008). 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). This work suggests that tropical insects are more likely to lose as climate temperatures increase and insects in temperate regions, like North America, could win.

As temperatures rise, the growing season in northern latitudes will resemble adjacent southern latitudes with growing seasons that begin earlier in the year and end later. For losing insects that cannot tolerate increasing temperatures in their current environment, occupying these north-shifting thermal conditions through shifts in the geographic range of the population could allow them to win and those insects unable to shift their geographic rage could lose. Winning insects could experience a net increase in both population size and geographical distribution with more individuals spread across more geography. Or, winning insects might experience a northern shift of their entire geographical distribution with no change in population size. In Europe, this has 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 shifts farther north and warmer days increase in frequency and duration, the spatial distribution of winning insects could track those favorable temperatures to utilize the more frequent more disperse thermal conditions. While warming northern latitudes do offer winning insects the opportunity to expand their populations into new geography, simply shifting into northern latitudes that resemble the thermal environment of adjacent southern latitudes could turn those winners into losers.

Temperature has a strong influence on insect growth and performance and in temperate regions, insects have evolved to synchronize their life histories with seasonal changes in temperature to maximize their growth during warmer seasons using photoperiod. At the equator, photoperiod, the number of daylight hours in a 24-hour period, is relatively constant at approximately 13 hours daily, however in the temperate regions, farther from the equator, photoperiod increases incrementally by latitude and season (Hut et al. 2013). During 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 have evolved to use photoperiod as a cue that approximate seasonal changes in temperature at specific latitudes despite the daily changes in temperature, which can vary annually. Warmer temperatures in temperate regions usually marks the availability of resources, like host plants for phytophagous insects, insects need to grow and develop. If an insect With growing seasons beginning earlier and ending later, a hypothetical photoperiod of 13 hours that previously indicated the last week of the growing season could, as temperatures increase, indicate the last two weeks of the growing season. Increasing temperatures are uncoupling photoperiod from changes in seasonal temperature and those insects that have evolved to approximate these changes using photoperiod could be impacted as seasonal temperatures rise. As temperatures increase, losing insects will be unable to adapt to these changes in the relationship between photoperiod and seasonality and they will fall further out of synchrony with the warm growing season. Those insects that win could have the capacity to adapt to the changes in photoperiod as it relates to changes in seasonal temperatures.

Broadly, adaptive capacity includes both the evolutionary changes and the phenotypically plastic ecological responses of insects to their environment.

As temperatures increase, existing genotypic and phenotypic variation in some insect populations could be selected for and that existing variation could allow them to adapt to and survive changes they face in their current environment or new environments. for and that variation (Parmesan et al. 1999). (Discuss selection and adaptation of climate specific traits, dessication, migration, species colonization paper could be a good reference\*\*\* )Those insect populations able to adapt to the local changes in their environment and/or colonize these new environments, may express greater plasticity in those traits that increase their active temperature range than those species whose ranges will shrink due to the shifting seasonality and warmer temperatures associated with climate change. Phenotypic plasticity is defined as an organism’s capacity to express different traits depending on the environment it encounters (Agrawal 2001). Specifically, phenotypic plasticity is when a single genotype within a species can express multiple different values for a particular phenotype. Because all environments vary, even if only temporally, organisms in those varying environments must be able to compensate for stress induced by this environmental variation. In general, all organisms possess at least a degree of phenotypic plasticity in some traits (Phillips et al. 2008, Price and Sol 2008). As organisms expand their range into novel environments (previously uninhabited geographical ranges or previously uninhabited temperature ranges), those genotypes that are to be successful must adapt to these novel environments and could do so through plasticity.

Adaptation, as a response to the temporal changes in an insect’s seasonal environmental (i.e. temperature and photoperiod), will be a function of selection pressures acting upon traits related to morphology, physiology, and behavior, including phenotypic plasticity in these traits (Lee 2002). In temperate regions, temperatures in the fall and winter are increasing across all latitudes while photoperiod is remaining relatively constant. Many insects synchronize their life history with these temporal changes in their environment to maximize their growth when temperatures are suitable and resources are available, using photoperiod allows these insects to approximate those changes. As temperatures rise and northern latitudes begin to resemble adjacent southern latitudes, insects will begin to colonize more northern geography.

Weather predicts short term changes in rain, humidity, temp, etc across short periods of time. Climate is an average weather factors across relatively longer periods of time.

Organisms acoss latitudes use predictable cues to sync their life history with the environ. Those

In the northern hemisphere at the height of

However, as latitude increases away from the equator, photoperiod is reduced. Those insects who depend on photoperiod to make life important history decisions will need to adapt to the changing photoperiod as they begin to colonize more northern environments and they could do so through plasticity in the vary traits that link their life histories to changes in their environment. The pitcher plant mosquito, *Wyeomii smithii,* illustrative how increasing temperatures have permitted northern range expansion and how plasticity can function to maintain synchrony with a novel environment. Pitcher plant mosquitos spend their larval growing phase entirely in the leaves of a pitcher plant. As photoperiod decreases, these insects enter a state of programmed dormancy in preparation for lower temperatures and declining resources. Rising temperatures have allowed these mosquitos to colonize more northern latitudes and plasticity in their response to photoperiod have resolved to allow these mosquitos to fully utilize these more northern resources. Between the years 1972 and 1996, the critical photoperiod (that is the threshold day length required to induce photoperiod among 50% of a population) has reportedly decreased form 15.79 hours of day light to 15.19 hours. This decrease in the number of daylight hours required to induce this dormancy response correlates to an increase in this insects growing season by an average of 9 days (Bradshaw and Holzapfel 2001). While photoperiod remains a crucial proxy for annual resource availability, the genotype controlling when this dormancy programming is initiated is phenotypically plastic and this plasticity allows these mosquitos to respond to changes in the environment.

Bradshaw 2001 paper: mosquito pops monitored over time. Winter is shrinking, important photoperiods are becoming more southern like. That is to say mosquitoes in the north that used to enter diapause at longer day lengths (northern fall/winter) are now entering diapause at shorter day lengths (southern fall/winter) because the growing season is longer. This is important because photoperiod in these mosquitoes is genetically determined fixed within populations. This change in diapause timing points towards selection acting on this population.

Agricultural systems are currently under perennial pressure from a throng of phytophagous pests that damage crops and reduce yields and to manage these populations, growers utilize a combination of, cultural, biological, and chemical tools to minimize the effects of these pests. However, as temperatures increase and insect pests “win” the tools we currently use to manage these pests will be additionally stressed. An unlucky scenario would be that increased temperatures could distribute insect pests into new agricultural geography or those warmer temperatures would increase the phenology of these pests, producing more crop damaging generations annually.

**Plasticity in Dormancy:** Insects are constantly monitoring their external, as well as internal, conditions and using that information to make life history decisions. Generally, insects respond to the stress of depleted environmental resources (food, water, suitable temperatures, or even other environmental parameters) through dormancy. Dormancy resulting from an immediate response to stress (quiescence) is a temporary state of reduced activity and one that can occur at any point during the life history of an insect. Alternatively, diapause is a type of dormancy that pre-empts reduced availability of resources, is genetically programmed, and while diapause may occur at any point during an insects life history, the life stage sensitive to the initiation of diapause within a species is consistent (Bale and Hayward 2010). Across different species, the genotype responsible for the pleiotropic effects of diapause is variable and the initiation of this genetic programming can be either obligate or facultative.

Diapause, across different insect species, can occur at almost any life stage, however within a single species, the timing of diapause is genetically determined and tends to be fixed along with the stage that is sensitive to the external cues that commit an insect to diapause. It is unclear wheather the genetic architecture of diapause responses is similar or different among species that differ in their diapause life history.

Diapause is a genetically inherited stage that can either be obligate or facultative. Obligate diapause is a genetically programmed part of an insects developmental trajectory that cannot be avoided (Koštál 2006, Hut et al. 2013). Facultative diapause is an environmentally programmed developmental trajectory, that can be induced or avoided depending on the cues an insect receives, or does not receive, from its environment. However, once initiated, diapause is defined as being a life history trajectory that is genetically predetermined and photoperiod is generally both necessary and sufficient in to induce the diapausing phenotype across many species of insects (Koštál 2006). For those insects that whose diapause is facultative, photoperiod is a relatively common cue used to approximate seasonal timing of resource availability because it is predictability variable across large time scales. It is during an insects sensitive period, the genetically predetermined point during an insect’s life history when they are sensitive to photoperiod, when they are physiologically competent to respond to changes in photoperiod by shifting away from direct development and towards diapause (Koštál 2006, Bale and Hayward 2010). During diapause, many insects suppress their metabolism, reduce their respiration, and suspend development to conserve energy and reduce energy consumption. Those insects that diapause feed very little or not at all during diapause and many do not feed after diapause ends, thus it is imperative that these insects begin diapause with enough resources to survive this stressful period and that they manage the resources they have stored to meet the requirements for metamorphosis and reproduction after diapause ends (Hahn and Denlinger 2007, Sinclair 2015). Accumulating enough resources, prior to their decline in the environment, is paramount if an insect is to survive the energetic demands of diapause.

**Storing Energy:** Diapause is a multistage biological state consisting of pre-diapause, diapause, and post-diapause development. Pre-diapause is demarked by the perception of some external cue like photoperiod or temperature by an insect during a genetically determined period during its life history known as the sensitive period (Koštál 2006). The perception of this external cue induces the genetic programming that destines an insect for diapause. Once diapause is induced, some diapause destined insects enter a preparation phase, and it’s during this phase when some insects can experience differences in physiology and behavior to promote diapause survival (Koštál 2006). During autumn, diapause is induced in adult monarch butterflies, *Danaus plexippus*, and diapause induction alters their behavior. As part of the diapause program in this species, they become migratory and begin their journey to overwintering sites in California and central Mexico where they will complete diapause before flying back to the southern US to begin reproduction the next spring (Goehring and Oberhauser 2002). In other insects like the mosquito *Culex pippens* or the Colorado potato beetle, *L. decemlineata*, diapause induction can dramatically change an individual insect’s physiology and in preparation for diapause, these insects accumulate large quantities of lipid compared to their non-diapausing conspecifics and storing these lipids as a source of energy during diapause (Hahn and Denlinger 2007, Bale and Hayward 2010, Sinclair 2015). In contrast, for some insects like the (insect citation), diapause preparation does not alter the amount of resources accumulated from its environment, instead consumed resources are directed away from reproductive tissues and somatic tissue development and toward storage (citation). The genes controlling the initiation of traits related to the diapause phenotype in most species represent a black box that requires more investigation to understand the mechanisms by which these genes function to initiate the many aspects of the diapause phenotype, downstream of their activation by photoperiod. The resulting phenotype generated from these genes is, generally, a combination of external and internal changes in character state, a phenotype that exists as a spectrum but is specific within a single species (citation). For some insects, diapause is a protective state where metabolic rates are drastically reduced to conserve energy and maintain physiological processes necessary to surviving diapause and thriving post-diapause (citation). For these insects, high energy biological molecules are the substrate that power the biological reactions allow these insects to thrive both during and after diapause, and they must be stored prior to the onset of diapause (citation). In preparation for diapause, some insects experience a steep increase in the stored amounts of lipids and proteins, specifically triglycerides and multimeric proteins, stored and produced by the fat body. While these molecules are biologically multifunctional, they also serve as energy reservoirs. Triglycerides, and other lipids, are used to stabilize membranes, slow or prevent desiccation, can be degraded into carbohydrates for energy. Stored proteins can serve as a reservoir of amino acids that can be reconfigured, under the right conditions, into other metabolically metabolic tools. These molecules have been observed to occur in high concentrations at the outset of diapause in (insect, insect, insect) (citation). Tracking the movement of these molecules using radiolabeled atoms, researchers show triglyceride carbons incorporated into (tissue, tissue, tissue) and amino acids from stored proteins incorporated into (tissue, tissue, tissue) (citation). Diverting resources away from direct development and into storage is a risky endeavor. Diapause preparations, in some species, is initiated during times when environmental resources are abundant. If seasonal temperatures vary away from historical averages and towards a warmer winter, physiologically switching away from direct development and preparing for diapause could be detrimental to the survival of a species (citation). Photoperiod is generally the proximate cue that insects use (within their specific latitudes) to initiate these changes in physiology because of its annual consistency and inherent relationship with changes in temperatures. Excluding the poles and the equator; as latitudes increase, photoperiods shrink and temperatures reduce gradually setting up a gradient of daylight hours during the growing season such that the photoperiod and temperatures experienced during the season becomes shorter and cooler (Hut et al. 2013). Historically, the relationship between photoperiod and temperature has predictably cycled from season to season, and it is in this way that insects, and other animals, have evolved to alter their phenotype to protect themselves from stressful changes in their environment. Ecologically, this regular pattern in changes in daylight hours sets up predictable species gradients of insects that are optimized to respond appropriately to the proximate stimulus in preparation for the eventual changes in their environments (Hut et al. 2013). In the context of increasing temperatures, higher latitudes experience the same photoperiod but the temperatures experienced during these photoperiods more resembles lower latitudes. Effectively leading to the uncoupling of photoperiod and temperature (Bale and Hayward 2010). understand the degree to which this uncoupling will disrupt species diversity and how pests are managed will require a model organism sensitive to these changes not unlike *Ostrinia nubilalis* (European corn borer).

If climate is causig longer longer and shorter, animals should increase their growing and initiate dormancy later

**European Corn Borer:** European corn borer, *Ostrinia nubilalis,* is an important agricultural pest here in the United States, its range extends from the Atlantic coast to the Rocky mountain range, and as far north as Canada and its diapause phenotype is facultative induced by both photoperiod and temperature. During its ultimate larval stage,

and photoperiod. seasonal climates and global food security are tenuously bound making a comprehensive approach to dealing with these changes imperative. Farmers and growers must be able to make short-term and long-term management decisions concerning methods, timing, and tools to utilize when planning pest control strategies and climate patterns are an important part of that calculus. with based upon how the climate affects those populations.

The consequences of increased temperatures on insect phenotypes can be estimated by understanding the direct relationship between latitudinal changes in temperature, photoperiod, and how insect respond to these changes physiologically.

Here in the United States, 92 percent of all the corn acreage is planted with a genetically engineered corn crop that expresses *Bacillus thurengensis* (Bt) crystalline protein toxin. Bt toxin was developed agriculturally to assist in managing European corn borer corn pest. pressure manage the that can be done to corn by an infestation of European corn borer. For this technology to be effective, farmers need to predict European corn borer infestations (Fernandez-Cornejo et al. 2014). “Studies detailing diapause-associated changes in intermediary metabolism and feeding physiology are needed across taxa with different diapause strategies to expand our understanding of the metabolic processes underlying prediapause reserve accumulation. The goal in this area is to under- stand the underlying neurological and endocrine signaling mechanisms that regulate diapause-associated shifts in feeding patterns and intermediary metabolism.” (unfinished)

**OBJECTIVE**

The objective of this study will be to quantify and compare energy stores between two genotypically different strains of *Ostrinia nubilalis,* the European corn borer. Further, European corn borer (ECB) destined for diapause and ECB avoiding diapause will be compared within each strain. Characterizing the energy stores of ECB destined for diapause, has yet to be capitulated in ECB and is necessary to understanding diapause biology and usefulness as a model system, managing it as an agricultural pest, and predicting its behavior as seasonal climates become less predictable (Denlinger 2008).

These Higher than average temperatures can lead to increased feeding, mating, and generation output. (example in corn) With climate being unpredictable and allow some insect pests to produce more generations during the season and Crop pests are able to produce more generations not only extend the growing season for plants it also extend the amplify the destructive effects of insect pests can is amplified and insect move into new regions or as especially those invasions that hold ecological or agricultural importance. (define invasions in significant terms and provide an agricultural example in corn). The largest threat posed by corn insect pests is in part a function of population turnover.

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

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

Characterizing these metabolic intermediates is intended to approximate the amount of energy an individual has reserved after feeding ends. European corn borer was chosen as the model for these experiments due to their facultative diapause life history strategy, differing genotypes and physiologies, and their different phenotypes. When either strain is exposed to the same photoperiodic and thermal cues in the laboratory, their specific response can be reproducibly observed but the physiological link between genotype and phenotype has not yet been described.

Approximately, $10 billion dollars is spent annually on chemical insecticides to control the damaging effects of insect pests (Pimentel 2005). Corn is an incredibly valuable crop in the United States and protecting it from actively feeding phytophagous insects includes the dynamic use of chemicals and biotechnology. To control the ephemeral outbreaks of ECB, farmers in the US spend approximately $10 billion dollars on chemical pesticides*.* In 2016, 92% of the corn acreage in the US was planted with BT corn. This type of pest management is a very powerful tool due to its specificity for phytophagous insects. However, the widespread use of this toxin pressurizes competition in the population. Those individuals in that can survive the toxic effects are given a mating advantage over its less advantaged, or dead, peers. Combined with the ability of ECB to produce one or two large generations a year, resistance across populations can be quickly amplified.

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

**PROPOSED METHODOLOGY**

**Origin and Husbandry of European Corn Corer.** The univoltine (UZ) and bivoltine (BE) strains of European corn borer (ECB) were generously provided courtesy of Dr. Dopman laboratory at Tufts University. These laboratory reared colonies were established in the year (\_\_) as larvae. These laboratory colonies will be continuously reared at 26C and a day cycle regimen of 16 hours of light and 8 hours of dark. These larvae have been sibling mated since their capture in (\_\_) and throughout the course of these experiments. To compare the differences in energy storage between diapause destined and diapause avoiding larvae, newly hatched larvae from each colony will be reared at 23 C and under two different lighting treatments. Larvae reared at 23 C with 12 hours of light and 12 hours of dark will diapause and larvae reared at 23 C with 16 hours of light and 8 hours of dark will avoid diapause (\_). Those diapause destined larvae from UZ and BE colonies will be labeled UZ12 and BE12 respectively. Those UZ and BE colony larvae reared under diapause avoiding conditions will be labeled UZ16 and BE16 respectively. Under these conditions larvae will be reared gregariously from hatching, through the 4th instar.

**Sampling Wandering Larvae.** Wandering larvae will be sampled using a modified version of the framework put forth in the 1982 study by Gelman and Hayes. For the purposes of this experiment, the wandering stage of ECB will be determined developmentally as the stage when the larvae stop feeding, the contents of their gut is cleared, and they search for refuge in preparation for either diapause or pupation. Feeding caseation, gut clearing, and seeking refuge will each be used as proxies for the beginning of the wondering stage. A 5th instar larva will be individually placed into an arena with food and allowed to eat ad libitum for 5 consecutive days. Starting on the 5th day, the burrowing larva will still be provided with food ad libitum, but every 24 hours it will be separated from the food source and placed at the opposite end of the arena. Feeding cessation will be determined by the larva not returning to the food source, or the larva not eating the food source. Gut clearing will be determined when frass production is replaced by the production of liquid from the anus or there are no anal exudates. Seeking refuge will be determined by the larva walking around the arena or the larva producing a sealed silken gallery. Any combination of these behaviors will be categorized as “wandering”(Gelman and Hayes 1982). Once the developmental stage is determined to be wandering, each larval sample will be weighed, hemolymph will be extracted for protein analysis, and the larva will then be freeze dried for lipid analysis. Larvae samples will be analyzed in cohorts of 9 for protein and lipid analysis.

**Protein Extraction and Quantification:** The hemolymph from each sample larvae will be extracted and the concentration of the total protein content in the extracted hemolymph will be measured. Extracting the lymph fluid will be done through a small incision along the cuticle of the prologs (Gelman and Woods 1983). Approximately 12 µL lymph fluid will be gathered into a pipet tip and stored into a microcentrifuge tube containing 500 µL of 1x PBS and 5 µL of Halt™ protease inhibitor. Sample larvae will be separated into cohorts and the protein concentration of those samples will be quantified using the Bradford protein assay. A cohort will consist of two larvae from each of the four treatment groups (UZ16, BE16, UZ12, and BE12). The Bradford assay utilizes the color shifting property of Coomassie Blue when it is bound to protein, the shifted color absorbs light at 595nm. The amount of light this molecule absorbs can be quantified. Bovine serum albumin dilutions of known concentration will be quantified using the Bradford assay. The measured absorbance of these dilutions of known protein concentration can be used to produce a linear relationship between absorbance and protein concentration. Dilutions of the lymph fluid of unknown concentration will be simultaneously quantified using the Bradford assay. The absorbance values at these dilutions will be compared to the dilutions of known concentration using linear regression.

**Protein Separation and Identification:** The extracted hemolymph proteins will be separated along a size gradient using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Once the protein concentration has been quantified using the Bradford analysis, a known concentration of protein will be added to each well in the gel and a 100mV electrical current applied to the gel for approximately two hours. These conditions will separate the proteins along a mass gradient and once separated, the migrated proteins will be stained with Bio-Safe™ Coomassie Stain, photographed, and the relative protein densities will be compared against a protein standard. The character of these protein will be determined using 2D-electrophoresis.

The separated proteins will then be identified using the facilities

**Triglyceride Extraction and Esterification:** Preparation for the extraction and esterification will begin with separating the larvae into cohorts. A cohort will consist of five larvae from each of the four treatment groups (UZ16, BE16, UZ12, and BE12) and four null samples. The null samples will serve to characterize the background effects of the extraction method. To capture the efficiency of the extraction and esterification method, (\_) will be used as a spike-in standard. Two of the four null samples will receive a known amount of the spike-in standard. By comparing the weight of the spike-in at the start of the extraction process to the recovery amount after the chromatographic analysis (explained later) a percent yield can be calculated. Before the triglycerides can be extracted or esterified, the dry weight of the larvae will need to be obtained after a period of lyophilizing. Dryness will be assumed when a larva does not lose more than 1% of its mass over a 24-hour period. The total lipid content of each of these larvae will then be extracted using a modification of the 1957 Folch and Sloane Stanley method (Folch et al. 1957). This modified method will allow for the partitioning of lipids using solvents of different densities. Each larva will be pulverized in a 2:1 solution of dichloromethane and methanol at a rate of 20:1 solution volume to larva volume. The dichloromethane will discriminately solubilize the less polar lipids that make up the larvae and methanol will trap the more polar molecules. To reduce any oxidative effects of oxygen, 0.05mg/mL of BHT (butylated hydroxytoluene) will be added to methanol. The resulting solution is decanted and saved. Dichloromethane will then be added back to the pulverized tissue, the tissue will again be pulverized and the resulting dichloromethane solution decanted and saved. This process will be repeated a total of three times. The saved solution of dichloromethane and methanol should contain the target triglycerides, along with non-target lipids, more-polar compounds, and solid some tissue, these non-target species will need to be removed. A magnesium silicate solid phase extraction column (Florisil SPE) will be used to fractionate the extremely polar compounds and solid debris out of the saved solution. The strongly polar adsorbent will interact strongly with the extremely polar compounds in the saved solution and its tightly packed nature will impede the movement of solids through the column. The saved solution will be placed onto the Florisil SPE column and the column will be rinsed with dichloromethane and methanol at rates of 1:0, 95:5, 9:1, 1:1, and 0:1 resulting in a rinsed lipid mixture. The rinsed lipid mixture will be dried under nitrogen gas and the weight recorded. To separate the more-polar lipids away from neutral triglycerides, a 2:1 mixture of dichloromethane and methanol will be added back to the dry rinsed lipids. To form an interface layer between the dichloromethane and methanol in the rinsed lipid mixture, water will be added to the solution at a rate of 20% the solutions volume and the aqueous solvent decanted and discarded. To ensure the dichloromethane layer is water free, the layer will be dried first using sodium sulfate crystals then again under nitrogen gas. This neutral lipid extract will be weighed and the extract saved.

After extraction, the triglycerides in the neutral lipid extract will be converted into their respective fatty acid methyl esters (FAME). To accomplish this conversion, the neutral lipid extract will be methylated via base-catalyzed esterification (AOCS, Cyber lipid, Christie, Ichikara, bumble bee paper, unpublished work J. Beck lab). The neutral lipid extract will be heated in a solution of 10M methanolic potassium hydroxide for 15 minutes, the solution is then vortexed and cooled on ice. While still on ice, 12M sulfuric acid will be added to the mixture, the mixture vortexed and heated. After heating the mixture, exactly 1 mL of hexanes will be added, the solution will be vortexed, and the hexanes layer decanted for a total of 3 mL of hexanes. Finally, the 3mL FAME solution will be decanted into a vial containing hydroscopic sodium sulfate to remove any water introduced into the solution. The dry FAME solution will be stored at -80C until chromatographic analysis.

**Triglyceride Identification and Quantification:** Using an Agilent 7980B gas-liquid chromatographer coupled with flame ionization detection (GC-FID), derivatized triglycerides will be identified and quantified. GC-FID is a method to compare the extracted esterified triglycerides to a standard mixture of esterified fatty acids of a known concentration. To identify each of the compounds in the FAME sample extract, the compounds in the mixture will be separated on a 30-meter capillary column lined with a highly polar liquid substrate, DB-WAX. The amount of time each compound spends interacting with the substrate will be recorded as its retention time and the intensity of the ionization detected will be recorded as its abundance. Each molecule in the FAME sample extract will interact with the column for a specific amount of time and that retention time will be used to identify that compound. The quantity of that compound when ignited by the flame ionizer will be recorded an abundance value. This process will be repeated using a commercially validated standard mixture of esterified lipids of known concentration and identity. The measured retention times and abundance of the compounds in the FAME sample extract will be compared to the retention times and abundance of the compounds in the standardized mixture. Comparisons of retention time and abundance will be used to estimate the identity and the concentration of the esterified triglycerides in the extract respectively.

**Data Analysis:** Data will be analyzed in batches and the resulting data will be analyzed using multivariate analysis. This will allow for many variables to be compared and reduced simultaneously.

Sample size will be determined using the power analysis formula. (http://www.statmethods.net/stats/power.html) NOVA

For a one-way analysis of variance use

pwr.anova.test(k = , n = , f = , sig.level = , power = )

where k is the number of groups and n is the common sample size in each group.

For a one-way ANOVA effect size is measured by f where

  
Cohen suggests that f values of 0.1, 0.25, and 0.4 represent small, medium, and large effect sizes respectively.

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