**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 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 20th 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 21st century that will continue 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 rate corresponds to the environmental temperature it experiences. Higher temperatures increase metabolic rate and lower temperatures reduce metabolic rate. Increased growing season temperatures for ectothermic insects could increase their metabolic rate, speed up their growth, and possibly shorten the development time to reproductive maturity. Mature adults that occur earlier in the growing season could increase adult population sizes and even 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 chemical insecticides.

Crop losses due to insect pest insect damage here in the United States from 1945 to 2000, has 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 under 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, our 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 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 distribution with more individuals spread across more geography. Winners could also experience a northern shift of their entire geographical distribution with no change in population size. In Europe, changes in range distributions 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 insects could exceed their thermal maximum and eventually cause mortality. Winners could have a wider thermal breadth 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. Being able 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. Those insects that experience distribution 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 their internal condition and the external environment, then and respond to changes in those environments as they occur. They 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, could impact growth, reproduction, or survival. Common environmental stresses for insects include extreme temperatures, ice, desiccation, and reductions in the availability of food. Generally, dormancy is a state of metabolic and developmental suppression used by many insects to mitigate the effects of both acute and chronic seasonal stress they encounter in their environment (Koštál 2006). As temperatures rise, winning insects could express phenotypic plasticity or experience evolutionary adaptations in their dormancy strategy to adjust to the shifting landscape of seasonally stressful environments 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 acute environmental stress (Koštál 2006). Once the stress is relieved (provided the exposure was not too extreme) quiescence is reversed and the insect’s activity can quickly resume. Seasonal temperature change is a common chronic stress insects typically encounter in their environment. To avoid or mitigate the consequences of chronic environmental stress many insects use diapause. For most temperate insects, when temperatures decrease maintaining a suitable metabolic rate for continued development becomes challenging. Further, as resource availability declines, they struggle to acquire enough energy to fuel metabolism, growth, and development. Diapause is one way insects can protect themselves from these chronic seasonal stresses. However, unlike quiescence, diapause is generally induced preemptively well before the 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 (Koštál 2006). By monitoring environmentally consistent cues that cycle with seasonality insects can reliably predict and prepare for seasonal changes in temperature and resource availability by using diapause to protect themselves.

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). Traits that mark diapause are genetically determined and typically highly heritable, but diapause timing, and development can and does vary from species to species and even among populations within a species. The diapause developmental trajectory has three distinct stages: pre-diapause, diapause, and post-diapause. Before diapause can be induced, that 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 during diapause however, most insects do not feed. The induction of diapause preempts seasonal reductions in resource availability giving insects the opportunity to accumulate the resources they will need to survive diapause while resources are available (Koštál 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 diapause. After diapause ends insects must have enough resources remaining to meet the anabolic requirements for development, metamorphosis, repair, and reproduction after diapause ends (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 suppression of metabolic activity and insects stop feeding (Tauber and Tauber 1981, Koštál 2006, Hahn and Denlinger 2007, Sinclair 2015). During diapause maintenance the endogenous mechanisms that support the diapause phenotype persist and diapause continues (Koštál 2006). Diapause termination is marked by the relief of those endogenous factors that initiate and maintain diapause, allowing development to resume under permissive conditions (Koštál 2006). After diapause is terminated, the potential to resume development exists, however, many insects do not immediately resume direct development. Instead, these insects can remain quiescent with their development arrested by environmental factors like low temperatures after diapause is terminated so they are ready to resume development as soon as environmental temperatures become favorable to do so (Koštál 2006).

Using diapause to synchronize an insect’s life history with available resources is crucial. Diapause is an alternative life history trajectory that requires an insect to monitor environmental cues, halt their development and suppress metabolic activity. 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. Developmental arrest and metabolic suppression can produce profound behavioral and physiological changes. The mechanisms controlling these changes are endogenously regulated and typically genetically heritable.

In temperate regions, warm temperatures persist in the spring and summer. During the spring and summer food and water are available and insects utilize these resources to develop and reproduce. As temperatures decline in the fall and winter, resource availability declines. For insects in temperate regions, remaining active at low temperatures can greatly reduce metabolic activity. Consequently, low metabolic activity reduces growth and can eventually cause mortality. Insects in temperate regions generally predict the indirect effects of seasonal changes in temperature 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 will increase. With growing seasons beginning earlier and ending later some of the seasonal cues insects use to predict changes in their environment, like photoperiod, may remain relatively consistent. Longer growing seasons will decouple the predictions of environmental cues and seasonal changes. Environmental cues that previously signaled the end of the growing season would underestimate the end of the 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.

Bradshaw and Holzapfel’s (2001) work concerning changes in diapause phenology through critical photoperiod shifts in the pitcher plant mosquito, *W. smithii,* provide one example of how insects could adjust to the increase in the number of warmer days and longer growing seasons through evolutionary adaptation as temperatures increase. Critical photoperiod is the number of light hours required to induce diapause in 50% of a population. In *W. smithii* 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, these insects 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 incrementally 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 diapause genotype 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 evolutionary 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.

**Modeling the Descent into Diapause:** Insects avoiding low winter temperatures in temperate regions, must meet the energetic demands of their metabolism during and in some cases after diapause. In preparation for diapause, some insects accumulate large amounts of lipids, amino acids, and/or carbohydrates. Lipids, specifically triglycerides, are the predominant source of metabolic energy used during diapause (Arrese and Soulages 2010, Hahn and Denlinger 2011). Triglycerides can be accumulated directly from an insects’ 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. These large protein complexes function as amino acid reservoirs. During diapause as metabolic proteins accumulate damage or are destroyed, the amino acids in hexamerins can be mobilized and used to repair or replace damaged proteins(Burmester 1999, Hahn and Denlinger 2007). After diapause, hexamerin proteins can 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).

In preparation for prolonged low temperatures and the absence of environmental resources, some insects accumulate and store more lipids to fuel their metabolism. For example, diapausing *Culex pippens* female mosquitos reared at 22°C and under a 14-hour photoperiod accumulate significantly more lipids in preparation for diapause relative to their non-diapausing conspecifics reared at the same temperature and under a 9-hour photoperiod. These stored lipids are utilized as a source of energy during diapause (Mitchell and Briegel 1989). In other insects, diapause preparation can 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 growing season temperatures increase, begin earlier and last longer, insects will experience warmer temperatures over a longer period. In the summer, warmer temperatures will increase metabolic rate. Increased metabolic activity will require more energy fuel an elevated metabolism. How will an increased rate of metabolism effect pre-diapause preparations and ultimately diapause survival? An elevated metabolism could require more nutrients than the insects can reasonably accumulate or if the insect can accumulate enough resources, increased metabolic activity could reduce stored energy to meet the demands of an active insect. Losers could enter diapause with an energy deficit, unable to properly fuel their metabolism these insects may not survive. However, insects that are capable of accumulating, and storing more nutrients during pre-diapause to support their increased metabolism could be winners as climate changes.

**You need a section stating that we need to understand how differences in over wintering demands may affect nutrient storage in the diapause preparatory period. One way to do that is to study natural polymorphism in diapause length within species. Then introduce ECB. Then get to your objective.**

The objective of this study is to determine how diapause induction alters the accumulation of resources in preparation for diapause. Specifically, the degree to which triglycerides accumulate in the fat body and hexamerin proteins accumulate in the hemolymph of genetically different strains of *Ostrinia nubilalis* (European corn borer, ECB) exposed to photoperiods that induce diapause compared to non-diapausing insects exposed to photoperiods that do not induce diapause. Characterizing these metabolic intermediates is intended to approximate the amount of energy an individual has stored in preparation for diapause.

European corn borer was chosen because it is an important insect pest here in the United States and within this species exist, at least, genetically distinct strains that exhibit different diapause phenotypes but occur sympatrically. Under controlled laboratory conditions, the unique response of each strain can be reproducibly observed and this thesis will serve to provide the physiological link between genotype and phenotype that has not yet been described. The univoltine-Z (UZ) strain genotype expresses a relatively long diapause phenotype, while the bivoltine-E (BE) strain genotype expresses a shorter diapause phenotype. The distinct strains of ECB have persisted as inbred siblings, originating from naturally occurring populations from Albany, New York genotype can either express or avoid diapause.

I hypothesize that the quantity of triglycerides and hexamerin proteins stored by the European corn borer stores in preparation for the additional stress of diapause, can be approximated by diapause the length of diapause. Specifically, diapause induced in univoltine-Z strain ECB larvae will accumulate and store more triglycerides and more hexamerin storage proteins to compensate for their longer and thus more metabolically demanding diapause period when compared to the shorter diapausing bivoltine-E ECB strain We predict that ECB preparing for a longer period of diapause will store significantly more energy in the form of triglycerides and hexamerins. ECB preparing for a relatively shorter diapause period will store relatively less triglycerides and hexamerins. While the non-diapausing treatments will store even less energy because they do not have the added metabolic cost of diapause.

European corn borer, *Ostrinia nubilalis,* is an important agricultural pest here in the United States, its current range extends into most of states east of the Rocky Mountains, as far north as Canada and, at times, as far south as Florida (Capinera 2000, Bohnenblust and Tooker 2010). As temperatures increase and insect pests “win” the tools we currently use to manage these pests will be additionally stressed. Higher than average temperatures can lead to increased feeding, mating, and generation output. With climate changes and temperatures rise, some insect pests to produce more generations during the season and Crop pests can 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.

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. Its diapause phenotype is facultative induced by both photoperiod and temperature.

During its ultimate larval stage, and photoperiod.

Investigating the energy requirements of this tenuous life history decision could expose the controlling factors of diapause phenology. Strategies that can precisely affect the progression of the 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 European corn borer (ECB) that will be used in this experiment were collected by members of the Dr. Dopman 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 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 16-hour photoperiod that promotes continuous development. To test for differences in stored triglycerides and storage proteins between diapause and non-diapause larvae, newly hatched larvae from each strain will be reared at 23°C under a 12-hour photoperiod to induce diapause or 16-hour 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 treatment from the UZ and BE strains will be hatched at 23°C and 65% relative humidity. These hatched larvae will be provided European corn borer diet, purchased from Frontier Agricultural Sciences, ad libitum. Non-diapause treatment larvae will experience a photoperiod of 16-hours, while diapause treatment larvae will experience a 12-hour photoperiod. The 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 32-well bioassay trays purchased from Frontier Agricultural Sciences, these trays will serve as individual arenas. 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 European corn borer larvae undergo in preparation for either diapause or pupation and adult metamorphosis (Gelman and Hayes 1982). Because the termination of frass production is indicative of the wandering phase, it 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 30 minutes. Those larvae whose gut is not clear will produce frass and will be placed back into their arenas and those that do not produce frass will be characterized as wanders, 30 individuals from each treatment will be collected, accessioned, and tracked for the duration of the experiment. Hemolymph and lipid extractions from sampled larvae will be analyzed for storage protein and triglyceride content, respectively.

**Protein Extraction and Quantification:** A portion of hemolymph will be drawn from larval samples and the total protein concentration in the extracted hemolymph will be measured. To extract hemolymph, a small incision will be made through the cuticle of the larval proleg (Gelman and Woods 1983). Using a micropipette, lymph fluid will be gathered and stored in a microcentrifuge tube. Extracting lymph from live larvae exposes the lymph fluid and the contained proteins to degradation from proteolytic enzymes. To reduce the activity of these enzymes, extracted hemolymph samples will be stored in microtubes containing 500 µL of 1x PBS and 5 µL of Halt™ Protease Inhibitor Cocktail with EDTA and kept at -80°C. After collecting lymph from larvae across each of the four treatments, samples will be grouped into cohorts, a sample of hemolymph will be taken from each individual larvae and total protein concentration will be quantified, separately. A cohort will consist of equal numbers of larvae from each strain, and from each photoperiod treatment. Hemolymph proteins will be quantified in relation to a standard curve of bovine serum albumin (BSA) using the Pierce™ Coomassie (Bradford) Protein Assay. When bound to protein, the coomassie-dye molecule experiences a shift in its conformation that changes the wavelength of light absorbed by the molecule from 465nm to 595nm. The total amount of light absorbed by this molecule when bound to a protein standard at known concentrations can be quantified using a spectrophotometer. The relationship between the wavelength of light absorbed by coomassie-dye bound to known protein concentrations can be used to infer the concentration of proteins in the hemolymph sample when bound by coomassie-dye given its measured absorbance.

**Storage Protein Separation and Quantification:** Storage proteins are multimers composed of six identical or similar subunits and each subunit weights approximately 80kDa each (Burmester 1999, Pick et al. 2009). The relative quantity of storage proteins in each larval sample will be determined by comparing the optical density of the larval samples to optical density of a known protein standard. To make this comparison, larval hemolymph and the protein standard will be separated by size using gel electrophoresis. 100ng/mL aliquots of each hemolymph sample will be mixed with sodium dodecyl sulfate, giving each protein in the mixture a net negative charge. The protein standard, containing a mixture of proteins of known size and concentration, will then be loaded onto a polyacrylamide gel, along with the larval lymph samples. Polyacrylamide is a synthetic matrix of composed of differently sized openings that selectively allow molecules to pass through the openings based on the size of the molecules. When a positive charge is applied to the gel, it attracts the negatively charged proteins and pulls them through the pores of the gel matrix based upon size. To visualize the ending location of the protein on the gel, Bio-Safe™ Coomassie Stain will bind proteins nonspecifically and the resulting color will be photographed and analyzed using the NIH ImageJ software.

**Lipid Extraction, Separation and Quantification:** The total lipid content from each larva will be extracted and quantified individually. First, larval dry mass will be determined by removing water from the larval sample through freeze-drying them in a vacuum at -80°C until their dry weight varies by less than 1% over a 24-hour period. Once dry, lipids 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 chloroform 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 are captured in the less polar chloroform layer. This layer will be decanted away from the remainder of the sample, the solvent removed and the total amount of lipids extracted from the sample can be quantified gravimetrically. The total lipid content extracted from each larval sample contains a mixture of different lipid classes from which triglycerides will need to be separated and quantified. Separating and quantifying triglycerides in the total lipid extract will be accomplished using Liquid Chromatography (LC) coupled with an Evaporative Light Scattering Detector (ELSD). LC takes advantage the physical properties of lipid molecules to adsorb to stationary phase that lines a column and interact with a mobile phase. The stationary phase is a matrix of C-18 silica gel. 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 C:18 silica matrix. Over time, the concentration of the mobile phase shifts from 100% A to 100% B. As the gradient changes, classes of lipid molecules in the sample desorb from the column flow into the ELSD where they are nebulized, the solvent is evaporated and the amount of light scattered is computed into a response peak. The response peak output of the ELSD can then be quantified by comparing it to the response peak of a standard concentration of triglycerides. The triglycerides used to prepare the standardized mixture are commercially available. Tristeric acid and tripalmitic acid will be purchased from Sigma Millipore and triheptadecanoic acid from VWR.

**Lipid Identification:** To identify the fatty acid components of the triglycerides quantified by LC-ELSD, the triglycerides in the total lipid extract will need to first be converted into fatty acid methyl esters (FAMEs). Each cohort will consist of lipid samples from 12 individual larvae, 4 from each treatment. Larvae samples from within cohort will be esterified and analyzed individually. Blanks will be used to qualify the background effect of the esterification. The efficiency of the esterification will be determined using triheptadecanoic acid, a spike-in standard obtained from Sigma Millipore. Triglycerides in the total lipid extract 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 capped and heated solution will be vortexed for two minutes, then cooled on ice for five minutes. While still on ice, the vial will then be uncapped and 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 into the reaction vial to solubilize the FAMEs. The hexane layer will then be decanted and any water species formed by the esterification procedure will be precipitated out of solution using sodium sulfate. Identification of the methyl ester species will be accomplished using Gas-Liquid 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 and the FAME molecules desorb from the column based on their molecular composition and the inert gas carries them to the detector. At the detector, retention time is recorded and each FAME molecule is ionized and the intensity of ionization is recorded as a peak area. FAMEs will be identified in comparison to a 37 Component FAME Mix purchased from Sigma Millipore.

**Data Analysis:** Using the techniques mentioned above, we predict that larvae exposed to a 12-hour photoperiod will accumulate more triglycerides and more storage proteins in preparation for diapause. Additionally, between diapausing larvae the univoltine-Z strain larvae should accumulate more proteins and triglycerides over bivoltine-E strain larvae. Prior to the compilation of this proposal, preliminary investigations to quantify triglyceride accumulation have already yielded results in line with our predictions. These data suggest there is a significant difference between the total lipid content of diapausing and non-diapausing larvae (Df = 1, p = 1.06x10-7). One interpretation of this data could be that as these larvae perceive photoperiod changes in their environment they experience changes in their physiology to increase their lean mass to support additional resource storage in preparation for diapause. To support this initial result, replications of these experiments will need to be conducted. These and future measurements of storage protein and triglyceride from each of the four treatments will be expressed as concentrations. The initial hemolymph protein concentration and putative storage protein concentrations will be determined relative to an external standard of known proteins and at known concentrations. Total lipid 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 and storage proteins will be used to explore the significance of interactions between measured parameters. Some of these parameters include larval wet mass, lean mass, and dry mass, total lipid mass, temperature, and photoperiod. Investigating these interactions determine if there are interesting patterns and could help explain the variation we see in the lab and nature.

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