**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 dOceanic 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). Exceedingly conservative projections of future temperatures estimate 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 temperatures increase, warmer temperatures will last longer into the fall and start sooner in the spring, effectively increasing the summer season and decreasing the winter season (Bradshaw and Holzapfel 2006, Hahn and Denlinger 2011, Scriber 2014).

An increase in the number of warmer days represents a net increase in favorable conditions for insect pests to thrive. For ectotherms and poikilotherms their metabolic rate (and by relation their growth and development) is directly proportional to external temperatures and increased temperatures can corresponds to increased metabolic rate. As it relates to insects, longer summers could sustain metabolic activity longer into the year, metabolic activity that can be directed towards more resource gathering, finding mates, or reproduction (Bradshaw and Holzapfel 2006, Scriber 2014). Because of the metabolic effect of warm temperatures on insects, their growth, in temperate regions, is synchronized with these warmer temperatures. As temperatures rise, longer, warmer growing seasons could increase the rate that some insects develop and reproduce and ultimately insect populations during the warmer seasons could increase (Bale et al. 2002). For insect pests, managing the potentially damaging effects caused by larger insect pest populations requires an integrated approach that includes the use of costly insecticides. In the United States, pesticide use is a large part of managing insect pest populations. In 2008, the cost to protect 21 major crops (including corn, soybean, and cotton) from pest insect damage using chemical pesticide application reached $12 billion dollars and 516 million pounds of pesticide (Fernandez-Cornejo et al. 2014). Pesticide use can manage insect pest populations, but even under strict application regimens insects can significantly reduce crop yields. In the United States starting in 1945 and ending in 2000, crop losses due to insect pest damage has nearly doubled from 7% to 13% and pesticide use has increased 10-fold (Pimentel and Burgess 2005). As summer temperatures begin earlier in the year and end later insect pests could respond to these longer growing seasons with increased development, leading to larger insect pest populations, earlier in the year and lasting longer. The cost to manage larger and earlier pest insect populations chemically is likely to increase substantially and these populations could result in lower crop yields (Culliney 2014). Lower crop yields will put into jeopardy access to safe nutrient rich foods for developed and developing countries around the world. Here in the United States the human 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). Under current climate conditions, yield reductions in chemically managed, pre-harvest crops due to arthropods is estimated between 13%-16% annually and understanding how crop yield may change due to insects requires further study. Investigating the responses of pest insect populations to increases in temperature is an opportunity to better understand and predict how climate change could effect 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:** As temperatures continue rise, there will be some insects that lose and other insects that win. Insects that experience a decrease in fitness will “lose” while other insects that experience an increase in fitness will “win”. Hypothetically, winners could be those insects whose viable egg production is directly proportional to the temperature, warmer temperatures for these insects would mean more viable eggs and more successful offspring. Alternatively, insects whose viable egg production is indirectly proportional to temperatures would lose, warmer temperatures for these insects would mean less viable eggs and less successful offspring. This distinction between losers and winners can be expressed as a function of fitness in the context of higher temperatures. The rate that temperatures are rising presents a biological hurdle for those insects currently existing on the margin of their behavioral, ecological, or physiological plasticity. Generally, extinction events are likely to affect insect populations that evolved in the context of highly predictable environments, like the ecological predictability experienced in the Arctic (Parmesan et al. 1999, Scriber 2014). Extrapolated temperature data, compiled from ice cores and other proximate sources representing the past 400 years, indicate that Arctic temperatures prior to 1840 were anomalously cold and post-1840 to 20th century, warmed by an average of 1.5°C across the arctic (Overpeck 1997). Currently, temperatures in the Arctic are rising at a rate nearly double that of temperate regions and the confluence of these increasing temperatures with other abiotic factors are predicted to have a more dramatic effect on organisms that thrive in the Arctic (Høye et al. 2007). In the Arctic, snowmelt provides flora and fauna with biologically available water and marks the beginning of the growing season. Like the other plants and animals in the Arctic, arthropods rely on flowing water for their very survival and, as such, are sensitive to the changes in their environment that signal the presence of flowing water. During the years between 1996 and 2005, the date of snowmelt has shifted by an average of 14.6 days. Tracking the shift in snowmelt date, 12 arthropod taxa have also shifted their date of emergence by 14.5 days during the same period (Høye et al. 2007). As Arctic temperatures warm, snowmelt date will shift earlier into spring these arthropods will emerge earlier. This shift in phenology is likely unsustainable and will push these arthropods to the limits of their phenotypic plasticity and genetic architecture (Høye et al. 2007). Eventually, these arthropods will fall out of synchrony with their environment, lose access to resources and become extinct. The traits specific to the biotic and abiotic environment these marginal insects encounter have evolved over thousands of years and as such these organisms have limited phenotypic plasticity. The average fitness of these “losers” will decline as their environment becomes more variable, populations will decrease and if temperatures continue to rise, those losers will become extinct (Bradshaw and Holzapfel 2008, Williams et al. 2015). The Arctic provides a somewhat simplified view of the interactions between warming temperatures and phenotypic plasticity, but temperate insects and even tropical insects will also face some of these same challenges to their seasonal synchrony and they will have a range of ways of compensating for altered seasonality.

As favorable habitat for temperate insects shifts farther north, the spatial distribution of some insects could track those favorable temperatures, shifting their range northward, a behavior some insects could use to compensate for their reduced fitness in their current environment (Parmesan et al. 1999, Breed et al. 2012). Shifting of population ranges north could be complimented by either a shrinking or stable southern boundary. A shrinking southern boundary could be due to increased temperatures in those regions where these insects previously thrived. As temperatures across their southern range increase, some insects will be unable to survive and as a result populations in those southern regions will decline, shrinking the distribution of insects along the southern boundary. In Europe, this has been observed in 35 species of non-migratory butterfly species. Of these butterflies, 63% were observed to have shifted their range northward and 3% shifted their range south (Parmesan et al. 1999). While spatial redistribution through range shifting can reduce the impact of climate change on insect populations, these shifts in spatial distribution can disrupt food security. Agricultural systems are currently under pressure from a suite of phytophagous pests that damage crops and reduce yields. These systems could be additionally stressed as warmer temperatures push new pests into established agricultural systems. The damage caused by both invading and endemic phytophagous insect pests could lead to increased use of chemical pesticides and reduced crop yields that further destabilize national food security.

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 (e.g., 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. 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.

**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. Obligate diapause is a form of genetically programmed dormancy that does not require prompting by any external conditions but rather this type of diapause is part of a continuous development life history (Koštál 2006, Hut et al. 2013). Facultative diapause is also a genetically programmed developmental trajectory, however facultative diapause is not a requisite developmental stage and it is initiated by the animals cuing in on some external factor, such as photoperiod. 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 changes in 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).

**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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