**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. Annual global surface temperatures and regional land surface temperature averages categorize 2016 as the warmest year on the record (NOAA National Centers for Environmental Information 2017). In 2016, global surface temperatures and North American land surface temperature averages were 0.94°C and 1.86°C above the 20th century averages, respectively. Exceedingly conservative estimates of climate temperature predict a 1.5°C increase in global surface temperature by the end of the 21st century and beyond (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 also surpassed 20th century temperature averages (NOAA National Centers for Environmental Information 2017). The average increase in seasonal temperatures, specifically winter and spring in temperate regions, decreases the severity of winters and as springs become warmer sooner, the duration of winter decreases (Bradshaw and Holzapfel 2006, Hahn and Denlinger 2011, Scriber 2014). Reduced severity and duration of winter represents a net increase in favorable conditions for insect pests to thrive. Warm, shorter winters increases the time available for growth, development, and reproduction for insect pests (Bradshaw and Holzapfel 2006, Scriber 2014). In temperate regions, insect growth is synchronize with warmer temperatures such that increases in the duration of the warm growing season could increase the rate at which some insects develop and also the number of pest generations each growing season, and ultimately pest population sizes (Bale et al. 2002). Managing the damaging effects caused by the potential for 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 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 temperatures rise and spring and winter will contract, insects will respond to these longer growing seasons with accelerated growth rates, increased voltinism, and greater population sizes managing those pests using chemical pesticides will cost more and possibly decrease crop yields in the future. The population of humans here in the United States is predicted to exceed 450 million by the year 2100 and increases in number of people to feed will demand sustained or even increased crop yields (Melorose et al. 2015). Phytophagous insect pests currently account for nearly (x-%) of crop loss in (year) but as temperatures rise, the cost of managing these pests will increase. Investigating the response of insect pest populations to increases in temperature offers the opportunity to better understand the effects of climate change on species evolution but also is directly consequential to mitigating the effects of insect populations to food security.

**Response to Climate Change:** As temperatures continue rise, there will be some insects that lose and other insects that win. Insects which experience a decrease in fitness will “lose” while other insects that experience an increase in fitness will “win”. The distinction between losers and winners can be expressed as a function of fitness in the context of higher temperatures. The rate at which 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 of the past 400 years, indicate 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 and genotypic plasticity (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)

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 population ranges north is could be complimented by either a shrinking or stable southern boundary. Across the southern population range, some insects will be unable to colonize these warmer will die, thus shrinking the distribution of insects along the southern boundary. Still, some insect populations could be phenotypically varied enough to compensate for the increased temperatures and will continue to survive (Parmesan et al. 1999). In Europe, this has been observed in 35 species of non-migratory butterfly species. Of these butterflies, 63% are observed to have shifted their range northward and 3% shifted their range south (Parmesan et al. 1999). While spatial redistribution through range shifting does function to 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 endemic 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 would lead to an increased use of chemical pesticides, reduced crop yields and further destabilize national food security.

Those insect populations able to adapt to the local changes in their environment and/or colonize new environments through shifts in their spatial distribution, have the capacity to differentially express traits that compensate for changes in their environment. This plasticity in the expressed traits of insect populations is a function phenotypic bet-hedging. Evolutionary bet-hedging describes the trade-off between average fitness and variance of fitness of the traits expressed by individuals in a population (Philippi and Seger 1989). Plasticity, in the context of phenotypes, is defined as an organisms capacity express different traits depending on the environment it encounters (Agrawal 2001). Because all environments vary, even if only temporally, organisms in those varying environments must be able to compensate to survive, thus it can be assumed that all organisms are phenotypically plastic to some degree (Phillips et al. 2008, Price and Sol 2008). The degree to which a population is phenotypically plastic will determine its capacity to compensate for a changing environment. All things being equal, a population with more phenotypic plasticity will experience higher fitness in the context of a varying environment and is more likely adapt to the changing environment compared to a population with a higher average fitness (Philippi and Seger 1989). Overcoming the biological hurdle of increased temperatures, phenotypes that are to be successful must become adapted in the population to colonize these novel environments.

As invading insects move into these novel environments, selection pressure might shift away from dispersal and tolerance traits and towards traits that are suitable for adapting to the new environment (Lee 2002). Adaptation, as a response to environmental gradients (i.e. temperature and photoperiod), will be a function of selection pressures that commonly act upon traits related to morphology, physiology, or plasticity (Lee 2002). Once insects begin to colonize these novel environments, more constant selection pressures in these environments may favor phenotypic specialization and eventually lead to the evolution of fixed, more competitive phenotypes (Weinig 2000, Lee 2002). As winters become shorter and less severe, insect populations could begin to colonize northern latitudinal clines and exploit available resources thus increasing their growing season. Here in United States as winters warm, pitcher plant mosquitos, *Wyeomii smithii,* are under strong directional selection. Between the years 1972 and 1996, has reportedly increased by an average of 9 days, representing a shift in average phenotype as a function of rising temperatures (Bradshaw and Holzapfel 2001). Pitcher plant mosquitos enter a state of genetically programmed dormancy at the end of their growing phase to avoid periods when resources unavailable in their environment. One of the hallmarks of this genetically induced dormancy include reduced or terminated feeding, thus increasing the amount of time an individual has access to these resources before dormancy could impart some evolutionary advantage. These insects, and others alike, synchronize their life histories with optimal temperatures and resource availability, this extended growing period is the result of strong directional selection and a greater capacity to adapt in response to higher temperatures in their environment (Bradshaw and Holzapfel 2001).

**Plasticity in Dormancy:** Insects are constantly monitoring their external, as well as internal, conditions and use that information as a predicate for their life history decisions. Generally, insects respond to the stress of depleted environmental resources (air, water, or food) through dormancy and these cues can initiate an immediate or preparative response. Dormancy that is the immediate response to stress (quiescence) is a temporary state of reduced activity and 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). The genotype responsible for the pleiotropic effects of diapause varies from species to species but photoperiod (the hours of daylight in a 24-hour period) generally is both necessary and sufficient in to induce the diapausing phenotype across many species of insects (citation). Because of its consistent nature and historical association with resource availability, insects have synchronized their life histories with available resources using the annual changes in photoperiod as proxy for when to end their growing seasons and prepare for diapause dormancy (citations). During the critical photoperiod, the genetically determined point during a species’ life history when they are sensitive to photoperiod, a species is competent to respond to the changes in photoperiod by shifting away from direct development and towards diapause dormancy (citations). The diapause dormancy phenotype generally includes suppressed rates of metabolism, metamorphosis, and reproduction (citations). Accumulating enough resources, prior to their environmental decline, is paramount if an insect is to survive the energetic demands of diapause.

**Storing Energy:** As summers begin to extend further into fall and winter, diapausing insects, those at the proper developmental stage and physiologically sensitive to photoperiod, begin to initialize genetic programming that influences how they allocate resources they take in from their environment (Bale and Hayward 2010). In 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 represents 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, during which, 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). While there are few exceptions, insects preparing for diapause, experience a steep increase in the amount of lipids, specifically triglycerides, and multimeric proteins produced and stored by the fat body. These molecules are biologically multifunctional and function as more than energy reservoirs. Triglycerides, and other lipids, are used to stabilize membranes, insulate tissues to prevent cold injury, slow or prevent desiccation, can be degraded into carbohydrates for energy or used as a precursor for steroids and other signaling molecules. Stored proteins function as a biological reservoir of amino acids and under the right conditions, stored proteins can be reconfigured or its amino acids incorporated into other metabolically important molecular tools, that can then be exploited metabolically. 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 is initiated during times when resources are abundant and committing, genetically, to suspending development, slowing growth, and inhibiting reproduction could end 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. 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 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.

**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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