**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, insects synchronize their growth 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). If temperatures continue to rise and insect pest populations respond to the rise in temperature 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. Predictions for the growth of the human population here in the United States are expected to exceed 450 million by the year 2100 and increases in number of people to feed will demand sustained or even increased crop yields (Department of Economic and Social Affairs and Population Division 2015). Investigating the response of insect pest populations to increases in temperature is directly consequential to both managing pest insect populations and food security.

**Response to Climate Change:** In response to climate change, some insects will “lose” (experience a decrease in average fitness), while other insects will “win” (experience stable or an increase in average fitness). The rate at which temperatures are rising presents a biological hurdle for those insects currently existing at the margins of their behavioral, ecological, or physiological capacity. Traits, specific to the biotic and abiotic environment these marginal insects encounter, have evolved over thousands of years. The relative fitness of “losers” under a changing climate compared to a stable climate will decline, populations will decrease and if temperatures continue to rise, those losers will become extinct (Bradshaw and Holzapfel 2008, Williams et al. 2015). 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). Permafrost core samples from the Arctic indicate temperatures in there have fluctuated between (x) and (y), and rising at a rate of (x) every decade since (x year). Currently, temperatures in the Arctic are rising at a rate (x rate per time), 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.

As insects struggle to adjust to climate change, “winners” will be the species best able to compensate for these changes either by rearranging their spatial distribution or by adapting phenotypically to changes in their local environmental. As favorable habitat for temperate insects shifts farther north, the spatial distribution of some insects will track those favorable temperatures and their range will shift poleward (Parmesan et al. 1999, Breed et al. 2012). Shifting population ranges north 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 also be a highly disruptive force. Agricultural systems are now under pressure from insect pests and are especially susceptible to shifts population distributions. These systems could be additionally stressed if insect pest populations push new pests into established systems. Damage to plants by established pest populations and shifted populations would lead to an increased use of chemical pesticides, reduced crop yields and further destabilize national food security.

Phenotypic plasticity Those insects that are successful in shifting poleward Invading insects that are successful at increasing the northern range boundary do so at the expense of the endemic plant and insect populations already occupying that location.

Alternatively, insect populations could respond to climate change by adapting to the local changes in their environment through phenotypic plasticity. Phenotypic plasticity Organisms that are phenotypically plastic, that is the ratio of average fitness to Phenotypic plasticity increases an organisms ability to compensate for changes in its environment by way of genetic or phenotypic variation (Visser 2008, Bradshaw and Johnson 2014).

Organisms that

there is a trade off between average fitness and variance of fitness. higher levels of phenotypic or genotypic plasticity, they could compensate for changes in their environment. Winning in a warming climate requires that organisms adapt to the changing climate. Adaptating to a changing climate can be accomplished ecologically or physiologically. Organisms able to adjust to a changing climate in an ecological context could experience changes in their migration patterns or changes in the distribution on their range poleward. These types of shifts in range distribution (Parmesan et al. 1999)

shift their distribution of phenotypes. Modeling organismal fitness in the context of a changing climate could be understood using the context of a changing climate, This shift in phenotype distribution will have a increase in fitness when compared to the original distribution of phenotypes. The rate at which this shift in phenotype distribution occurs is the Plasticity is the rate that this shift towards an increase in average fitness occurs

The average temperature increases in a product of the increases in seasonal temperatures higher temperatures Higher temperature trends are also mirrored within seasons. of higher temperatures is also represented within seasons. As annual temperatures rise, seasonal temperatures are rising

Using these seasonal cues, insects have synchronized their life history such that they are able to take advantage of seasonally available resources and avoid seasons when those resources are unavailable. Understanding the effect of climate change on insect life histories has direct implications for insect pest management and food security. The consequence of these interactions, between climate change and the physiological response of insects, presents an interesting opportunity for scientific investigation, the results of which is of great importance as our nation and the world becomes more populated.

In agricultural systems, invading pest insects and less predictable climate during crop growing seasons can devastate crop yields. Increases in pest pressure Increasing the pest pressure to important agricultural commodities increases the production cost. 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 pestscan 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. Tracking hospitable climates through shifts in population range boundaries or adjusting to local temperatures physiologically by phenotypic plasticity are strategies employed by both migratory and non-migratory insect species (Somero 2010). However the degree to which these types of adaptations are possible hinges upon the range of phenotypic plasticity. The ability of an insect to adapt to changing climates is limited by its phenotype. Further, insects that are most vulnerable to extinction are those whose life histories and resource requirements are hyper-specialized.

Insects can either adapt to these changes and As seasons change from summer to winter, hours of day light decrease. Changes in daylight hours have been associated with shifting seasons for centuries and insects have used these seasonal cues as proxies for seasonal resource depletion. The interaction between climate change and insect physiology can be estimated by understanding the correlation between latidunal changes in temperature, photoperiod, and how insect respond to these changes physiologically. Because photoperiod is annually consistent, organisms rely on this abiotic factor to influence physiological changes in preparation for seasonal changes in resources. The pattern of photoperiod is regular and predictble across latitudes. Ecologically, this regular pattern in changes in daylight hours sets up predictable species gradients (clines). Species of insects that inhabit different clines,

Insects monitor these external conditions and predicate life history decisions upon these cues. In preparation for the seasonal shifts, insects see changes in their metabolism, rate of development and metamorphosis. Generally insect suppress the rate of their metabolism and development to conserve energy during this stressful period.

Populations are expected to rise from the 321.2 million to 398.3 million people here in the United States (POPULATION REFERENCE BUREAU 2015). Farmers depend on the predictive nature of these seasonal cues to determine when to plant, chemically treat, and harvest their field crops to meet the food demand of the nation. As temperatures warm, insect pests are expected to respond with increased growth rates, longer seasons, and increases in populations. These additional factors will stress an already stressed system and managing the effects of these pressures will necessitate the use of more chemical pesticides and result in increased crop losses.

**Dormancy Adaptations**

Dormancy is a protective state that insects can use when local environmental conditions become untenable. Many insects use some form of dormancy to adjust to unfavorable environmental circumstance or changes in climate. Insects generally resort to dormancy when resources like food and water become depleted or even absent. Quiescence is a form of dormancy that is an immediate response to changes (or cues) in an insects environment. In the case of quieseccence, the cue and the environmental change are singular and the insects phenotype changes immediately (definition root meaning in phenotype plasticity and provide example). In the case where the cue and the environmental change are separated in time, the insect responds to the cue Dormancy can also be preparative in that when an environmental cue is receicved the insect responds by shifting its physiology to This type of response can be a reaction to environmental changes, quiesecence, or the insect response can be in prepatation to impending environmental canges, diapause. While there are different levels of dormancy diapause is a category of dormancy that is initiated before resources or circumstance in the local environment change.

**Diapause and Food Security**

Food, mates, water, shelter; these are all resources that organisms must manage to be competitive and survive within their environment, so how is this accomplished? During periods of food abundance one can intuit how managing it can be understood. Simplistically, when available food concentrations are higher than the amount of food required for an organism to survive, they will either consume just enough resources to survive or they will over consume. The dynamics of survival during times of food scarcity is not as simplistic but can be understood in general terms. Overcoming resource decline in most cases leads to different types of dormancy but the intensity, preparation, and duration of that dormancy are all able to be modulated to meet the specific requirements of an organism’s current environment. Organisms experiencing environments with reduced resources can cope with these stressful periods by migrating to locations where resources more readily available. Another way organisms compensate for these resource poor times is by storing more resources during resource rich times to last through the stressful period.

Changes in food, water, oxygen, and temperature can all have direct effects on the immediate developmental state of an organism. Quiescent dormancy is an organisms immediate response to these types of environmental changes. This type of dormancy is not genetically predetermined and while it does reduce the activity of an organism, its metabolic activity is relatively constant (effect of chilling and reduced oxygen and how insects respond to these types of changes… useful and old example in drosophilla). Diapause is a state of dormancy that is initiated in advance of shifting environmental conditions. This type of dormancy is generally precipitates from environmental cues such as light or temperature. Diapausing insects use these environmental cues to initiate physiological changes that function as to protect the insect from the seasonal absence of resources. The genetic programming that is initiated when diapause is induced can result in the acquisition and storage of more resources necessary to survival. (unfinished)Predictable 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 decisions about when to control pest populations based upon how the climate affects those populations. 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 the difference in stored energy between the 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).

I hypothesize that the amount of energy a European corn borer stores in preparation for the additional stress of diapause, is related directly to how it performs during diapause. Specifically, diapause destined individuals preparing for diapause will increase their storage of triglycerides and storage proteins in direct 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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