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

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**MS Thesis Proposal**

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**Changing Climate:** Earth’s climate is warming. According to the National Oceanic and Atmospheric Administration, 2016 was the warmest year on the record with global surface temperatures and North American land surface temperatures averaging 0.94°C and 1.86°C above the 20th century averages respectively (NOAA National Centers for Environmental Information 2017). Additionally, conservative projections of future temperatures estimate at least a 1.5°C increase in global surface temperature by the end of the 21st century that will continue to increase thereafter (DeLucia et al. 2008, Stocker et al. 2015). Seasonal temperature averages in the United States during 2016 echoed this upward trend and average temperatures for spring, summer, fall, and winter all surpassed 20th-century temperature averages (NOAA National Centers for Environmental Information 2017). In north temperate regions of the continuous United States, for example in Maine, annual temperatures can peak in the summer around 24°C and in the winter temperatures frequently fall below freezing. As annual temperatures continue to increase, warmer days will begin earlier in the year and end later in the year, reducing the number of cool days in the spring and fall, effectively increasing the duration of the summer growing season (Bradshaw and Holzapfel 2006, Hahn and Denlinger 2011, Scriber 2014). For many organisms, warmer temperatures generally increase development, and for these organisms more frequent warmer days during the year could favor development during these longer warmer seasons. As it relates to insects these longer, warmer growing seasons could provide more time for development that could be directed towards more resource gathering, mate finding, or reproduction possibly leading to increased population sizes and even greater numbers of generations each year (Bale et al. 2002, Bradshaw and Holzapfel 2006, Hahn and Denlinger 2011, Scriber 2014). For insect pests, managing the potentially damaging effects caused by larger insect pest populations that last longer into the growing season will require an integrated approach and likely increased use of chemical insecticides.

Insecticide use can manage insect pest populations, but even under strict application regimens insects can significantly reduce crop yields. Under current climate conditions, yield reductions in chemically managed, pre-harvest crops due to arthropods is estimated to be between 13%-16% annually (Culliney 2014). Crop losses due to insect pest insect damage here in the United States from 1945 to 2000, has nearly doubled from 7% to 13% while insecticide use has increased 10-fold (Pimentel and Burgess 2005). As warmer temperatures begin earlier in the year and end later, larger pest insect populations could lead to lower crop yields and the cost to manage these potentially larger and earlier occurring pest populations using chemical insecticides is likely to increase. As temperatures continue to rise, lower crop yields due to insect damage will endanger access to safe nutrient-rich foods for people in developed and developing countries around the world. Here in the United States, our population is predicted to exceed 450 million by the year 2100 and this population increase will demand sustained or even increased crop yields (Melorose et al. 2015). Investigating the responses of pest insect populations to increasing temperature is an opportunity to better understand and predict how climate change could affect these pests, and use those predictions to mitigate their damaging effects and ensure the security of our nation’s food as populations increase.

**Responses to Climate Change:** Animal performance is influenced by the thermal conditions they experience in their environments, increased temperatures could affect animals either positively or negatively (Huey and Stevenson 1979, Chown and Terblanche 2006). An increase inseasonal temperatures in temperate regions will extend the duration of the warm growing season, with warmer days that arrive earlier in the spring and end later into fall. In effect, warmer seasonal temperatures in northern latitudes will resemble the seasonal temperatures of adjacent southern latitudes, increasing the geographic distribution of warmer environments (Parmesan et al. 1999, Breed et al. 2012). Insects whose populations are impacted negatively by climate change can be colloquially termed “losers” and those impacted positively can be termed “winners”. The direct and indirect interactions between temperature and the resulting winners could lead to increased temperature tolerance, increasing population size?, or expanding geographic ranges (Hughes 2000, Williams et al. 2008). Winning insects could adjust to warmer temperatures through plasticity or adaptation. Understanding how climate change might increase insect populations and expand population distributions, or how insects could adjust to warmer temperatures could help predict some of the damaging effects these winning pest insects could have on agricultural crops.

An insect’s body temperature directly affects its performance, and the effect of body temperature on performance can be described using a thermal performance curve (Huey and Stevenson 1979). At the peak of this curve is an insect’s thermal optimum, this is the temperature where performance is maximized. The range of temperatures where the performance of an insect is half of the thermal optimum represents the thermal breadth. Finally, the range of temperatures where performance is positive is an insect’s thermal tolerance range. Those temperatures at the edge of an insects thermal tolerance are termed the critical thermal maximum and critical thermal minimum, respectively (Bale et al. 2002, Huey et al. 2012, Sinclair et al. 2016). As warmer days begin earlier in the year and last longer, losing insects could be unable to tolerate these changes due to narrow thermal breadth. For these losing insects, warmer daily and seasonal temperatures could reduce their performance by exceeding their thermal breadth earlier in the day or earlier in the season. Continued increases in temperatures for these insects could be lethal by exceeding their critical thermal maximum. Winning insects, in contrast, could tolerate warmer temperatures due to a wider thermal breadth. Additionally, some winners whose thermal environment is currently below their thermal optimum experience increased performance as temperatures increase towards their thermal optimum. In a review of the effects of thermal conditions on population fitness (with fitness defined as the intrinsic population growth of r-strategy insects), Deutsch et al. (2008) tracked and compared population size between 38 representative insect species from temperate and tropical latitudes. For those representative species across temperate latitudes, the thermal breadth of these insects tended to be wider and the thermal conditions experienced in these locations, on average, tended to be further away from their critical thermal maximum compared to the representative taxa from tropical latitudes (Deutsch et al. 2008). In the tropics, environmental temperatures vary little relative to temperatures in temperate regions and insects in tropical regions experience temperatures that tend to be closer to their optimum temperature relative to temperate insects whose environment tends to be cooler than optimum. This suggests that tropical insects already exist near their thermal limits and thus could quickly become losers as climate warms.

As temperatures rise, the growing season in northern latitudes will resemble adjacent southern latitudes with growing seasons that begin earlier in the year and end later. For losing insects that cannot tolerate increasing temperatures in their current environment, occupying these north-shifting thermal conditions through changes in the geographic range of the population could allow them to win, and those insects unable to shift their geographic rage could lose. Winning insects could experience a net increase in both population size and geographical distribution with more individuals spread across more geography. Winning insects might also experience a northern shift of their entire geographical distribution with no change in population size. In Europe, changes in range distributions have been observed in 35 species of non-migratory butterfly species. Of these butterflies, 63% were observed to have a distribution shift northward and 3% were observed to have a distribution shift southward (Parmesan et al. 1999). As favorable thermal conditions for winning insects shift farther north and warmer days increase in frequency and duration, the spatial distribution of winning insects could track those favorable temperatures. Warming northern latitudes do offer winning insects the opportunity to shift their population distributions. However, those insects that experience shifted distributions will be exposed to environmental cues, like photoperiod, that are intrinsic to these northern latitudes. Photoperiod, like temperature, is an important environmental cue that insects use to make life history decisions. Failure to adjust to the photoperiods of these warmer northern latitudes could negatively impact the timing of life history events for those shifted populations, turning winners into losers.

Seasonal changes in temperature are cyclic and correspondingly can delimit the availability of resources (like host plants for phytophagous insects). For plants and animals alike, temperature has a strong influence on their growth and performance, but daily temperatures can fluctuate from year to year. To prepare for seasonal changes in temperature, many plants and animals synchronize their development using other environmental cues that consistently cycle with these changes in seasons. In the temperate regions farther from the equator, photoperiod consistently changes incrementally by latitude and season (Hut et al. 2013). During the summer, photoperiod is long and increases as latitude increases; while in the winter, photoperiod is short and decreases as latitude increases. Insects in temperate regions use these consistent, incremental changes in photoperiod at specific latitudes to synchronize their life histories with the availability of resources in their environment. With growing seasons beginning earlier and ending later in each year with climate change, a hypothetical photoperiod of 13 hours that previously indicated the average beginning of the growing season could, as temperatures increase, indicate on average the second week of the growing season instead. As temperatures increase, photoperiod will become uncoupled from seasonal changes in temperature and resource availability. Insects that depend on photoperiod to make life history decisions, but cannot adjust to the warmer temperatures approximated by photoperiod, could lose. Winning insect populations could be pre-adjusted to warmer temperatures or, as temperatures rise, they could gain the ability to adjust to the warmer predictions of photoperiod (Williams et al. 2008). The capacity to adjust to these photoperiodic changes could be the result of phenotypic plasticity or evolutionary adaptations.

As temperatures rise, it can directly affect the performance of insects. When environmental temperatures are too high, they can exceed the thermal maximum of insects by inhibiting activity, development, and eventually causing mortality. However, warmer and less predictable seasonal temperatures can also have indirect effects on insect performance by increasing environmental stress. Warmer and less predictable temperatures could lead to prolonged and intermittent changes in resource availability. Additionally, increased temperatures generally increase metabolic activity in insects. As their metabolic rate increases, insects will require access to more resources to fuel their elevated metabolism or they could starve. Winning insects could adjust to these cyclic and stochastic changes in their environment through phenotypic plasticity. Phenotypic plasticity is defined as the capacity of a single genotype to express multiple, different phenotypes as a function of the environmental conditions that the genotype encounters (Agrawal 2001). For example, phenotypically plastic insects could express traits that reduce their metabolic rate as temperatures increase. This phenotypic plasticity could mediate the effects of reduced resources increased temperatures by expressing phenotypes better suited to tolerate environmental stress. In a recent survey of phenotypic plasticity, researchers investigated the response of eight clinally distinct *Drosophila melanogaster* populations to determine if phenotypic plasticity could increase their resistance to starvation (Hoffmann et al. 2011). These populations were reared under temperature regimes that fluctuated daily, similar to the daily average summer and winter temperatures for 6 days with a spike in temperature for 5 hours on day 7. After day 7, the flies were treated under starvation conditions and mortality was tracked. In each of the populations, starvation resistance was significantly increased in those fly treatments exposed to summer temperature regimens compared to winter (Hoffmann et al. 2005). Temperature spikes, for these flies, reduces their reliance on environmental resources and increases their resistance to starvation. The phenotypically plastic response of flies to express traits that modulate the rate of their metabolism could help these insects win as climate changes and environmental resource availability fluctuates.

In temperate regions, insect phenology tracks seasonal changes in temperature because these seasonal changes determine the availability of resources. To predict seasonal changes in resource availability, many insects depend on photoperiod to synchronize their life history and maximize their development. As temperatures rise, the thermal conditions experienced in northern latitudes will begin to resemble adjacent southern latitudes, however, photoperiod in these latitudes will remain consistent. If insects are to win, they will need to adjust how they respond to the warmer temperatures in the context of photoperiod as a seasonal cue. Some insects could adjust to these changes through evolutionary adaptation. Evolutionary adaptation can be described as a product of natural selection changing the frequency of heritable traits within a population whereby genotypes within a population that are better suited for a given environment increase in frequency (Lee 2002). As temperatures warm, insects with genotypes that enable them to adjust to the warmer temperatures predicted by photoperiod could migrate into these changing northern regions that now have more southern thermal conditions, becoming climate change winners? Or something similar.

The relatively consistent nature of photoperiod makes it a reliable cue insects can use to approximate seasonal changes. If winning insects are to take advantage of the warmer, longer growing periods, they will need to adjust their life history by shifting their response to photoperiod cues through plasticity or evolutionary adaptation. Phenotypic plasticity and/or evolutionary adaptation could be how some insects adjust to the changing climate. A warmer climate means growing seasons will become longer and it will be those insects that are synchronized with these extended growing seasons that will have the advantage and could win.

**Adjusting through Dormancy:** To ensure their survival, organisms must monitor their internal and external environments and respond to changes in those environments as they occur. They must actively work to avoid conditions that become too stressful and take advantage of conditions that are favorable. Stress in an insect’s natural environment could be considered to be any condition that, if encountered, could impact growth, reproduction, or survival. Common environmental stresses for insects include extreme temperatures, drought, ice, and reductions in the availability of food. Environmental stress that occurs and over a relatively short period of time can be categorized as acute stress. While stress that occurs over a relatively prolonged period can be considered chronically stressful. Generally, dormancy is a state of metabolic and developmental suppression used by many insects to mitigate the effects of both acute and chronic stress they encounter in their environment (Koštál 2006). Insects are ectotherms and are readily susceptible to thermal stress. As temperatures rise, winning insects could adjust to stressful temperatures using dormancy.

As acute stress is perceived, some insects use quiescence to quickly respond to relatively short-term, stressful conditions. Quiescence is a transient state of reduced activity that insects can use to temporarily protect themselves from acute environmental stress (Koštál 2006). Once the stress is relieved (provided the exposure was not too extreme) quiescence is reversed and the insect’s activity can quickly resume. Insects also monitor their environment for chronic stress and some insects use diapause to avoid or mitigate prolonged environmental stress. Diapause is an endogenously regulated type of dormancy used by insects in response to predictable seasonal stress encountered in their environments (Koštál 2006). Seasonal temperature change is a common stress insects typically encounter that can indirectly affect resource availability in their environment. For most temperate insects, as temperatures decrease their physiology struggles to maintain a metabolic rate suitable for continued development. Further, as resource availability declines, they struggle to acquire enough energy to fuel their metabolism. Diapause is one way insects can protect themselves from these chronic seasonal stresses. However, unlike quiescence, diapause is generally induced preemptively well before the environment degrades and becomes stressful. By monitoring environmentally consistent cues, like photoperiod in temperate regions, insects can reliably predict seasonal changes in temperature and other stressors, protecting themselves by entering diapause.

Additionally, diapause synchronizes an insect’s life history with seasonal resource availability. In temperate regions, warm temperatures persist in the spring and summer. During the spring and summer food and water are available and insects utilize these resources to develop and reproduce. As temperatures decline in the fall and winter, resource availability declines. For insects in temperate regions, remaining active at low temperatures can greatly reduce metabolic activity. Consequently, low metabolic activity reduces growth and can eventually cause mortality. Diapause is one strategy insects use to avoid the stress of prolonged seasonal stress in their environments.

Diapause is a genetically regulated, environmentally influenced alternative developmental trajectory that is usually marked by metabolic suppression and arrested development in a specific life stage (Koštál 2006). The start of diapause usually precedes the chronic seasonal environmental stress, and the end of diapause does not necessarily correspond directly with the end of the environmental stress (Koštál 2006). This life history phase can be “obligatory” as observed in univoltine insect species. Univoltine insects experience environmental conditions that restrict reproduction to one generation each growing season. Or diapause can be “facultative” as observed in multivoltine insect species. The environmental conditions multivoltine insects experience permit more than one generation each growing season.

In general, insects that use diapause as a life history strategy depend on the timing of diapause to synchronize their life history decisions with resource availability. Using diapause to synchronize an insect’s life history with resources is crucial. If an insect enters diapause too late they could expose themselves to stressful environmental conditions and if diapause ends too soon the environment may not be suitable for that insect’s growth and development, or mates may not be available for reproduction. Diapause is an alternative life history trajectory that requires an insect to monitor environmental cues, halt their development and suppress metabolic activity. Developmental arrest and metabolic suppression can produce profound behavioral and physiological changes and the mechanisms controlling these changes are regulated differently between different species. Within a species, the traits that mark diapause are genetically determined and highly heritable, but diapause timing and development does vary from species to species. Within a single insect species the environmental cues that stimulate diapause, the life stages sensitive to those cues, and the resulting diapause phenotype are typically consistent (Bale and Hayward 2010). The diapause developmental trajectory has three distinct stages; pre-diapause, diapause, and post-diapause. Before diapause can be induced in an individual, that individual must reach a genetically determined sensitive period. During an insect’s sensitive period, it can perceive the environmental cue or cues that induce diapause and it is physiologically competent to respond to that cue or cues. During pre-diapause, the sensitive stage perceives the necessary environmental cue or cues, there is a shift away from continuous development and towards the diapause developmental trajectory. During diapause, most insects do not feed however, they insects must continue meet the energy requirements of their metabolism.

During pre-diapause, insects accumulate and store resources in preparation for the stress they will encounter during and after diapause to include cold temperatures, reduced access to food, ice, and desiccation. The induction of diapause preempts seasonal changes and gives insects the opportunity to accumulate the resources they will need to survive diapause while those resources are available (Koštál 2006). For many insects, the physiological changes that occur during pre-diapause can have substantial effects on their survival during diapause and even potentially affect post-diapause outcomes. In preparation for diapause, many insects accumulate and store resources in the form of lipids, proteins, and carbohydrates as sources of energy to fuel metabolism during diapause. It is imperative that insects accumulate enough resources to meet the energetic and anabolic requirements for development, metamorphosis, repair, and reproduction after diapause ends (Hahn and Denlinger 2007, Sinclair 2015) . Following the successful completion of pre-diapause, insects enter diapause, progressing through three distinct stages; initiation, maintenance, and termination.

Diapause initiation is generally marked by the suspension of continuous development and suppressed metabolic activity. During diapause maintenance the endogenous mechanisms that support the diapause phenotype persist and diapause continues (Koštál 2006). Diapause termination is marked by the relief of those endogenous factors that initiate and maintain diapause, allowing development to resume under permissive conditions (Koštál 2006). After diapause is terminated, the potential to resume development exists, however, many insects do not immediately resume direct development. Instead, these insects remain quiescent with their development arrested by environmental factors like low temperatures after diapause is terminated so they are ready to resume development as soon as environmental temperatures become favorable to do so (Koštál 2006).

As climate changes and average seasonal temperatures increase, some of the seasonal cues insects use to predict changes in their environment, like photoperiod, may remain relatively consistent as warm growing seasons begin earlier and end later. Longer growing seasons will decouple the predictions of environmental cues and seasonal changes. The environmental cues that previously signaled the end of the growing season will begin to underestimate the end of the growing season. Those insects that adjust to these underestimated predictions to resynchronize their lifecycles with the growing season, either by evolutionary adaptations or phenotypic plasticity in their response to these shifting environmental cues could win as climate changes.

The pitcher plant mosquito, *Wyeomii smithii,* illustrates how expanding growing seasons can lead to evolutionary changes in the timing of diapause initiation and termination within populations over time. Pitcher plant mosquitos spend their entire pre-adult life growing in the water-filled leaves of pitcher plants. These mosquitos inhabit temperate regions as far south as the Gulf of Mexico and as far north as northern Canada. Across this wide latitudinal range, these insects experience their longest growing seasons at the southern end of their range and increasingly shorter growing seasons at more northern latitudes. At the end of the warm growing season, photoperiod gets shorter. Once photoperiod drops below a genetically determined number of light hours, larvae perceive that cue and these larvae enter the larval diapause developmental trajectory. Critical photoperiod is the number of light hours required to induce diapause in 50% of a population. In *W. smithii* critical photoperiod for diapause induction is highly heritable. Bradshaw and Holzapfel (2001) sampled several populations of *W. smithii* larvae from latitudes between Florida and Canada in the years 1972, 1988, 1993 and 1996 and reared in a common garden laboratory setting under strict environmental control. Populations collected in 1972 and 1996 were exposed to incrementally different photoperiods to determine their critical photoperiod (Bradshaw and Holzapfel 2001). In 1972, the critical photoperiod of larvae populations collected at 50°N, averaged 15.79 hours while the critical photoperiod of larvae populations collected in 1996 at the same latitude averaged 15.19 hours. Because of the rigor with which these experiments were conducted and the highly heritable nature of diapause genotype within this species, these results suggest the populations collected in 1996 have evolved and are now genetically different than those collected in 1972. These northern mosquitoes, on average, are delaying diapause by approximately 9 days and this shift correlates with the average increase in the number of warmer days experienced in this region (Bale and Hayward 2010). Delayed diapause initiation could be evolutionary adaptive. For these insects, warmer temperatures are indirectly responsible for the increased availability of environmental resources these insects need to grow and develop. The mosquitoes that delay diapause initiation could access those resources and continue to grow, develop, and reproduce for an additional 9 days.

Bradshaw and Holzapfel’s (2001) work concerning diapause adaptation through critical photoperiod shifts in *W. smithii* provide one example of how insects could adjust to the increase in the number of warmer days, and longer growing seasons through genetic adaptation as temperatures increase. For some insects, warmer seasonal temperatures and longer growing seasons will increase the duration of resource availability. Those insects that can adjust to longer growing seasons without compromising the protection of diapause could be winners as climates change.

**Stored Resources and the Descent into Diapause:** In temperate regions, seasonal temperatures are geographically widespread and they predictably cycle between warm summers and cold winters. When temperatures are warm and resources like food and water persist, insects can grow, develop, and reproduce. When temperatures are low, environmental resources become limited and insect activity during the winter declines or is directly prohibited be cool temperatures. To avoid the prolonged stress of winter, many temperate insects use diapause. During diapause, insects generally experience suppressed metabolic activity, arrested development, and do not feed (Tauber et al. 1986, Koštál 2006, Hahn and Denlinger 2007, Sinclair 2015). Surviving diapause requires insects to protect themselves from stressful abiotic conditions like low temperatures and prolonged water loss. Additionally, insects must budget the energy they have accumulated and stored to fuel metabolism throughout the diapause period, which lasts months in many cases (Sinclair 2015). To meet the energetic demands of diapause and the time after diapause, some insects, in preparation for diapause, accumulate large amounts of lipids, amino acids, and or carbohydrates. Lipids, specifically triglycerides, are the predominant source of metabolic energy during diapause (need a ref for this type of statement). Triglycerides can be accumulated directly from an insects diet or synthesized in the fat body from amino acids or carbohydrate intermediates (Hahn and Denlinger 2007, Arrese and Soulages 2010). Amino acids are generally stored as hexamerins. These specialized proteins build up in the insect fat body or hemolymph prior to diapause. During diapause, these proteins function as amino acid reservoirs used to repair or replace damaged proteins. After diapause, hexamerin proteins can be catabolized into its constituent amino acids and the resulting amino acids can be used to build exoskeleton, repair damaged proteins, and build new tissues during morphogenesis. Carbohydrates are polymerized and stored as glycogen in the fat body or as trehalose in the hemolymph (Hahn and Denlinger 2007, Arrese and Soulages 2010).

In preparation for prolonged low temperatures and the absence of environmental resources, some insects accumulate and store more lipids to fuel their metabolism. For example, diapausing *Culex pippens* female mosquitos reared at 22°C and under a 14-hour photoperiod accumulate significantly more lipids in preparation for diapause relative to their non-diapausing conspecifics reared at the same temperature and under a 9-hour photoperiod. These stored lipids are utilized as a source of energy during diapause (Mitchell and Briegel 1989). In other insects, diapause preparation can lead to an increase in hexamerin storage, like the Colorado potato beetle, *Leptinotarsa decimlineata*. For this experiment, Colorado potato beetles were brought into the lab and reared under a 10-hour photoperiod to induce diapause and an 18-hour photoperiod to bypass diapause. From each of these treatments diapause protein 1 mRNA (the gene transcript that codes for a hexamerin storage protein) was extracted from these adult beetles and its concentrations compared. Beetles under diapause conditions were sampled on day 4 and day 6 after adult emergence, and 2 months into diapause (De Kort and Koopmanschap 1994). While mRNA from beetles not exposed to diapause conditions was extracted on day 1 and day 4 after adult emergence (De Kort and Koopmanschap 1994). Northern blot analysis comparison of hexamerin mRNA between diapause and non-diapause *L. decimlineata* beetles shows a significant difference in the accumulation of this hexamerin (De Kort and Koopmanschap 1994).

As temperatures rise, insects will be specifically taxed as their metabolic rate is proportional to external temperatures. Increase their metabolic rate and could allow these insects to grow faster during the warm season but how will an increased metabolism effect pre-diapause preparations and ultimately diapause survival? Losing insects could be morphologically or physiologically unable to accumulate and store enough nutrients during pre-diapause to meet their increased metabolic needs at increased temperatures. Losing insects could have fixed physical limits to their morphology, making the accumulation and storage of more nutrients unattainable. Or these losers could be metabolically limited, unable to metabolize enough of the resources they do accumulate into storage at a biologically acceptable rate. These losing insects could enter diapause with an energy deficit, unable to properly fuel their metabolism these insects may not survive. However, insects that are capable of accumulating, and storing more nutrients during pre-diapause to support their increased metabolism could win as temperatures increase and climate changes.

**European corn borer as a model:** 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, as far north as Canada and as far south as Florida (cite). 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. A changing climate will affect the However, as temperatures increase and insect pests “win” the tools we currently use to manage these pests will be additionally stressed. An unlucky scenario would be that increased temperatures could distribute insect pests into new agricultural geography or those warmer temperatures would increase the phenology of these pests, producing more crop damaging generations annually.

Extended growing seasons in the context of a changing climate could extend the duration of the environmental resources insects need to accumulate to survive diapause.

* + Pest control 🡪 exploit traits to disrupt diapause phenotype
  + Current pest
  + Clinal distribution indicative of adaptative radiation and response to increased temps directly and indirectly
  + Genetically distinct diapause phenotypes
    - Because diapausing phenology and genotype are heritable, ECB can
  + Modeling evolution of species

**Agricultural:** 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. Agricultural systems are currently under perennial pressure from a throng of phytophagous pests that damage crops and reduce yields and to manage these populations, growers utilize a combination of, cultural, biological, and chemical tools to minimize the effects of these pests. **Agriculture BT:** 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).

**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 lipids the 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 12-hour photoperiod 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-Z (UZ) and Bivoltine-E (BE) strains of European corn borer (ECB) that will be used in this experiment were collected by members of the Dr. Dopman laboratory at Tufts University. Strain identity was determined genotypically using the pgFAR autosomal gene, this gene codes an important enzyme involved in determining the female sex-pheromone blend, and is partly responsible for the strain differences (Lassance et al. 2010). Both strains were collected as larvae, pupae and adults from New York state prior to 2015 (Wadsworth et al. 2015). For the duration of the experiment, each strain will be continuously mass reared at 26°C under a 16-hour photoperiod. To compare the differences in stored triglycerides and storage proteins between diapause and non-diapause larvae, newly hatched larvae from each strain will be reared at 23°C under conditions that either induce diapause or non-diapause. Those larvae treated under diapause inducing conditions from the UZ and BE strains will be labeled UZ12 and BE12 respectively and those treated under diapause avoiding conditions will be labeled UZ16 and BE16 respectively.

**Sampling Wandering Larvae.** European corn borer eggs, intended for treatment, from the UZ and BE strains will be hatched at 23°C and 65% relative humidity. These hatched larvae will be provided European corn borer diet, purchased from Frontier Agricultural Sciences, ad libitum. Non-diapause treatment larvae will experience a photoperiod of 16-hours, while diapause treatment larvae will experience a 12-hour photoperiod. The regime experienced by each treatment will be held constant throughout the duration of the experiment. At the beginning of the fifth instar, larvae will be separated into 32-well bioassay trays purchased from Frontier Agricultural Sciences, these trays will serve as individual arenas. Once larvae reach the end of the fifth instar, they will be assayed to determine if they have entered the wandering phase. At the beginning of the wandering phase, larvae discontinue feeding, empty the contents of their gut and after clearing their gut larvae no longer produce frass. The wandering phase is a necessary step all European corn borer larvae undergo in preparation for either diapause or pupation and adult metamorphosis (Gelman and Hayes 1982). Because the termination of frass production is indicative of the wandering phase, it will be used to diagnose putative wandering larvae. To diagnose late fifth instar larvae as wandering, larvae will be removed from their individual arenas and held in a clean, empty petri dish and monitored for 30 minutes. Those larvae whose gut is not clear will produce frass will be placed back into their arenas and those that do not produce frass will be characterized as wanders, 30 individuals from each treatment will be collected, accessioned, and tracked for the duration of the experiment. Hemolymph and lipid extractions from sampled larvae will be analyzed for storage protein and triglyceride content, respectively.

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

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

**Lipid Extraction, Separation and Quantification:** The total lipid content from each larva will be extracted and quantified individually. First, larval dry mass will be determined by removing water from the larval sample by freeze-drying them in a vacuum at -80°C until their dry weight varies by less than 1% over a 24-hour period. Once dry, lipids will be separated from the larval tissues using a slightly modified Folch method (Folch et al. 1957). This method takes advantage of the polarity and density differences between chloroform and methanol that allow each solvent to selectively solubilize molecules of similar polarity and to produce distinct layers when mixed together. When a larval sample is solubilized in this solvent mixture, the less polar lipids are captured in the less polar chloroform layer. This layer will be decanted away from the remainder of the sample, the solvent removed and the total amount of lipids extracted from the sample can be quantified gravimetrically. The total lipid content extracted from each larval sample contains a mixture of different lipid classes from which triglycerides will need to be separated and quantified. Separating and quantifying triglycerides in the total lipid extract will be accomplished using Liquid Chromatography (LC) coupled with an Evaporative Light Scattering Detector (ELSD). LC takes advantage the physical properties of lipid molecules to adsorb to stationary phase that lines a column and interact with a mobile phase. The stationary phase is a matrix of C-18 silica gel. The mobile phase includes two solutions; mobile phase A is a 0.1% acetic acid in methanol mixture and mobile phase B is 40% hexanes in 2-propanol. Samples are injected onto the column and the contained lipids adsorb to the C:18 silica matrix. Over time, the concentration of the mobile phase shifts from 100% A to 100% B. As the gradient changes, classes of lipid molecules in the sample desorb from the column flow into the ELSD where they are nebulized, the solvent is evaporated and the amount of light scattered is computed into a response peak. The response peak output of the ELSD can then be quantified by comparing it to the response peak of a standard concentration of triglycerides. The triglycerides used to prepare the standardized mixture are commercially available. Tristeric acid and tripalmitic acid will be purchased from Sigma Millipore and triheptadecanoic acid from VWR.

**Lipid Identification:** To identify the fatty acid components of the triglycerides quantified by LC-ELSD, the triglycerides in the total lipid extract will need to first be converted into fatty acid methyl esters (FAMEs). Each cohort will consist of lipid samples from 12 individual larvae, 4 from each treatment. Larvae samples from within cohort will be esterified and analyzed individually. Blanks will be used to qualify the background effect of the esterification. The efficiency of the esterification will be determined using triheptadecanoic acid, a spike-in standard obtained from Sigma Millipore. Triglycerides in the total lipid extract will be methylated via base-catalyzed esterification with an acid catalyzed work-up (Christie and Christie 1993, Liu 1994). Extracted lipids will be mixed in a solution of 10M methanolic potassium hydroxide at 55°C for thirty minutes in a capped vial. The capped and heated solution will be vortexed for two minutes, then cooled on ice for five minutes. While still on ice, the vial will then be uncapped and 12M sulfuric acid will be added to neutralize the KOH and terminate the reaction. After the reaction is terminated 3 mL of hexanes will be added into the reaction vial to solubilize the FAMEs. The hexane layer will then be decanted and any water species formed by the esterification procedure will be precipitated out of solution using sodium sulfate. Identification of the methyl ester species will be accomplished using Gas-Liquid Chromatography (GC) coupled with a Flame Ionization Detector (FID). GC-FID separates each FAME by taking advantage of the specific interactions between different FAMEs and the packing material in a DB-WAX capillary column. The FAMEs in the sample adsorb onto the column and inert gas flows through the column. Over time, the column temperature increases and the FAME molecules desorb from the column based on their molecular composition and the inert gas carries them to the detector. At the detector, retention time is recorded and each FAME molecule is ionized and the intensity of ionization is recorded as a peak area. FAMEs will be identified in comparison to a 37 Component FAME Mix purchased from Sigma Millipore.

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

**REFERENCES:**

**Agrawal, A. A.** **2001**. Phenotypic Plasticity in the Interactions and Evolution of Species. Science (80-. ). 294: 321–326.

**Arrese, E. L., and J. L. Soulages**. **2010**. Insect Fat Body: Energy, Metabolism, and Regulation. Annu. Rev. Entomol. 55: 207–225.

**Bale, J. S., and S. A. L. Hayward**. **2010**. Insect overwintering in a changing climate. J. Exp. Biol. 213: 980–994.

**Bale, J. S., G. J. Masters, I. D. Hodkinson, C. Awmack, T. M. Bezemer, V. K. Brown, J. Butterfield, A. Buse, J. C. Coulson, J. Farrar, J. E. G. Good, R. Harrington, S. Hartley, T. H. Jones, R. L. Lindroth, M. C. Press, I. Symrnioudis, A. D. Watt, and J. B. Whittaker**. **2002**. Herbivory in global climate change research: Direct effects of rising temperature on insect herbivores. Glob. Chang. Biol. 8: 1–16.

**Bradshaw, W. E., and C. M. Holzapfel**. **2001**. Genetic shift in photoperiodic response correlated with global warming. Proc. Natl. Acad. Sci. 98: 14509–14511.

**Bradshaw, W., and C. Holzapfel**. **2006**. Evolutionary Response to Rapid Climate Change. Science (80-. ). 312: 1477–1478.

**Breed, G. A., S. Stichter, and E. E. Crone**. **2012**. Climate-driven changes in northeastern US butterfly communities. Nat. Clim. Chang. 3: 142–145.

**Burmester, T.** **1999**. Evolution and function of the insect hexamerins\*. Eur. J. Entomol. 96: 213–225.

**Chown, S. L., and J. S. Terblanche**. **2006**. Physiological Diversity in Insects: Ecological and Evolutionary Contexts. Adv. In Insect Phys. 33: 50–152.

**Christie, W. W., and W. W. Christie**. **1993**. Preparation of ester derivatives of fatty acids for chromatographic analysis. 69–111.

**Culliney, T. W.** **2014**. Crop Losses to Arthropod Pests, pp. 201–226. *In* Integr. Pest Manag. Vol 3.

**DeLucia, E. H., C. L. Casteel, P. D. Nabity, and B. F. O’Neill**. **2008**. Insects take a bigger bite out of plants in a warmer, higher carbon dioxide world. Proc. Natl. Acad. Sci. 105: 1781–1782.

**Denlinger, D. L.** **2008**. Why study diapause? Entomol. Res. 38: 1–9.

**Deutsch, C. A., J. J. Tewksbury, R. B. Huey, K. S. Sheldon, C. K. Ghalambor, D. C. Haak, and P. R. Martin**. **2008**. Impacts of climate warming on terrestrial ectotherms across latitude. Proc. Natl. Acad. Sci. U. S. A. 105: 6668–6672.

**Fernandez-Cornejo, J., R. Nehring, C. Osteen, S. Wechsler, A. Martin, and A. Vialou**. **2014**. Pesticide Use in U.S. Agriculture: 21 Selected Crops, 1960-2008.

**Folch, J., M. Lees, and G. H. S. Stanley**. **1957**. A simple method for the isolation and purification of total lipids from animal tissues. J Biol Chem.

**Gelman, D. B., and D. K. Hayes**. **1982**. Methods and Markers for Synchronizing Maturation of Fifth-Stage Larvae and Pupae of the European Corn Borer , Ostrinia nubilalis. Ann. Entomol. Soc. 75: 485–493.

**Gelman, D. B., and C. W. Woods**. **1983**. Haemolymph ecdysteroid titers of diapause-and nondiapause-bound fifth instars and pupae of the european corn borer, Ostrinia nubilalis (HÜBNER). Comp. Biochem. Physiol. 76A: 367–375.

**Hahn, D. A., and D. L. Denlinger**. **2007**. Meeting the energetic demands of insect diapause: Nutrient storage and utilization. J. Insect Physiol. 53: 760–773.

**Hahn, D. A., and D. L. Denlinger**. **2011**. Energetics of Insect Diapause. Annu. Rev. Entomol. 56: 103–121.

**Hoffmann, A. A., C. Sgrò, and M.** **2011**. Climate change and evolutionary adaptation. Nature. 470: 479–485.

**Hoffmann, A. A., J. Shirriffs, and M. Scott**. **2005**. Relative importance of plastic vs genetic factors in adaptive differentiation: Geographical variation for stress resistance in Drosophila melanogaster from eastern Australia. Funct. Ecol. 19: 222–227.

**Huey, R. B., M. R. Kearney, A. Krockenberger, J. a M. Holtum, M. Jess, and S. E. Williams**. **2012**. Predicting organismal vulnerability to climate warming: roles of behaviour, physiology and adaptation. Philos. Trans. R. Soc. Lond. B. Biol. Sci. 367: 1665–79.

**Huey, R. B., and R. D. Stevenson**. **1979**. Intergrating thermal physiology and ecology of ecotherms: a discussion of approaches. Am. Zool. 19: 357–366.

**Hughes, L.** **2000**. Biological consequences of global warming: is the signal already apparent? Trends Ecol. Evol. 15: 56–61.

**Hut, R. A., S. Paolucci, R. Dor, C. P. Kyriacou, and S. Daan**. **2013**. Latitudinal clines: an evolutionary view on biological rhythms. Proc. Biol. Sci. 280: 20130433.

**De Kort, C. a. D., and A. B. Koopmanschap**. **1994**. Nucleotide and deduced amino acid sequence of a cDNA clone encoding diapause protein 1, an arylphorin-type storage hexamer of the Colorado potato beetle. J. Insect Physiol. 40: 527–535.

**Koštál, V.** **2006**. Eco-physiological phases of insect diapause. J. Insect Physiol. 52: 113–127.

**Lassance, J. M., A. T. Groot, M. A. Lienard, B. Antony, C. Borgwardt, F. Andersson, E. Hedenstrom, D. G. Heckel, and C. Lofstedt**. **2010**. Allelic variation in a fatty-acyl reductase gene causes divergence in moth sex pheromones. Nature. 466: 486–491.

**Lee, C. E. E.** **2002**. Evolutionary genetics of invasive species. Trends Ecol. Evol. 17: 386–391.

**Liu, K.-S.** **1994**. Preparation of fatty acid methyl esters for Gas-Chromatographic analysis of lipids in biologcal materials. J. Am. Oil Chem. Soc. 71: 1179–1187.

**Melorose, J., R. Perroy, and S. Careas**. **2015**. World Population Prospects: The 2015 Revision, Key Findings and Advance Tables. Working Paper No. ESA/P/WP.241., United Nations, Dep. Econ. Soc. Aff. Popul. Div.

**Mitchell, C. J., and H. Briegel**. **1989**. Inability of diapausing Culex pipiens (Diptera: Culicidae) to use blood for producing lipid reserves for overwinter survival. J. Med. Entomol. 26: 318–26.

**NOAA National Centers for Environmental Information**. **2017**. State of the Climate: Global Climate Report for Annual 2016. (https://www.ncdc.noaa.gov/sotc/national/201613).

**Parmesan, C., N. Ryrholm, C. Stefanescu, J. K. Hill, C. D. Thomas, H. Descimon, B. Huntley, L. Kaila, J. Kullberg, T. Tammaru, W. J. Tennent, J. a Thomas, and M. Warren**. **1999**. Poleward shifts in geographical ranges of butterfly species associated with regional warming. Nature. 399: 579–583.

**Pick, C., M. Schneuer, and T. Burmester**. **2009**. The occurrence of hemocyanin in Hexapoda. FEBS J. 276: 1930–1941.

**Pimentel, D.** **2005**. Environmental and economic costs of the application of pesticides primarily in the United States In Integrated Pest Management: Innovation-Development Process. Environ. Dev. Sustain. 7: 229–252.

**Pimentel, D., and M. Burgess**. **2005**. Environmental and economic costs of the application of pesticides primarily in the United States. Integr. Pest Manag. 3: 47–71.

**Scriber, J. M.** **2014**. Climate-driven reshuffling of species and genes: Potential conservation roles for species translocations and recombinant hybrid genotypes, Insects.

**Sinclair, B. J.** **2015**. Linking energetics and overwintering in temperate insects. J. Therm. Biol. 54: 5–11.

**Sinclair, B. J., K. E. Marshall, M. A. Sewell, D. L. Levesque, C. S. Willett, S. Slotsbo, Y. Dong, C. D. G. Harley, D. J. Marshall, B. S. Helmuth, and R. B. Huey**. **2016**. Can we predict ectotherm responses to climate change using thermal performance curves and body temperatures? Ecol. Lett. 19: 1372–1385.

**Stocker, and V. B. and P. M. M. (eds. . T.F., D. Qin, G.-K. Plattner, M. Tignor, S.K. Allen, J. Boschung, A. Nauels, Y. Xia**. **2015**. Summary for Policymakers. Clim. Chang. 2013 - Phys. Sci. Basis. 1542: 1–30.

**Tauber, C. A., and M. J. Tauber**. **1981**. Insect seasonal cycles: genetics and evolution ,~4195. 12: 281–308.

**Tauber, M. J., C. A. Tauber, and S. Masaki**. **1986**. Seasonal adaptations of insects, Ecology.

**Wadsworth, C. B., X. Li, and E. B. Dopman**. **2015**. A recombination suppressor contributes to ecological speciation in OSTRINIA moths. Heredity (Edinb). 114: 593–600.

**Williams, S. E., C. Moritz, L. P. Shoo, J. L. Isaac, A. a Hoffmann, and G. Langham**. **2008**. Towards an Integrated Framework for Assessing the Vulnerability of Species to Climate Change. PLoS Biol. 6: e325.